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PROVISIONAL APPLICATION COVER SHEET [37 CFR 1.53(c)]

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TITLE OF THE INVENTION (280 characters max)

MOLECULAR SIGNATURES AND ASSAY FOR FLUOROQUINOLINE RESISTANCE IN BACILLUS ANTHRACIS

APPLICANT(S) STATUS UNDER 37 CFR § 1.27

_X_ Applicant(s) and any others associated with it/them under § 1.27(a) are a SMALL ENTITY

ENCLOSED APPLICATION PARTS

_6_ Specification (number of pages)
_____ Drawings (number of sheets)
_____ Assignment
_X_ Other (specify): Appendices A through D

FEE AND METHOD OF PAYMENT

_X_ A check for the filing fee of $ 80.00 is enclosed.
The Commissioner is hereby authorized to charge any fees under 37 CFR 1.16 and 1.17 which may be required by this filing to Deposit Account No. 03-1728. Please show our docket number with any charge or credit to our Deposit Account. **A copy of this letter is enclosed.**

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

_X_ No ____ Yes, the name of the U.S. Government agency and the Government contract number are:

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PROVISIONAL APPLICATION FILING ONLY

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PATENT

PROVISIONAL APPLICATION

of

Paul S. Keim
Talima Pearson
Lance Price
and
Joseph D. Busch

For

UNITED STATES LETTERS PATENT

on

MOLECULAR SIGNATURES AND ASSAY FOR FLUOROQUINOLINE RESISTANCE IN BACILLUS ANTHRACIS

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49212/CAB/A599 **Express Mail No. EL818387304US**

MOLECULAR SIGNATURES AND ASSAY FOR FLUOROQUINOLINE RESISTANCE IN *BACILLUS ANTHRACIS*

SUMMARY OF THE INVENTION

The preferred therapeutics for human anthrax infections is the fluoroquinolone, ciprofloxacin (CIP). In order to characterize fluoroquinolone resistance in *Bacillus anthracis*, we used an *in vitro* stepwise selection procedure to isolate spontaneous high-level ciprofloxacin (CIP) resistant mutants. We were able to isolate *B. anthracis* mutants with minimum inhibitory concentrations (MICs) as high as 64 μg CIP/ml (1000 fold increase over the wild-type MIC). We discovered that first-level mutants, selected on 0.25 μg CIP/ml, developed 1 of 5 missense point mutations in the Quinolone Resistance Determining Region (QRDR) of the *gyrA* gene. The most common of these mutants (Ser 85 -> Leu) was used in a second selection on 1.5 μg CIP/ml. Second-level mutants developed 1 of 4 missense mutations in the QRDR of the *parC* gene. The most common second-step mutant (Ser 81 -> Phe) was used in a third and final selection on 24 μg CIP/ml. Two strains isolated from the third step had additional missense point mutations in the QRDR of the *gyrA* gene. However, all other third level isolates were devoid of mutations within the four known QRDRs and exhibited no evidence of increased multi-drug efflux.

We have defined the molecular basis for fluoroquinolone (CIP in particular) action and thus provide the molecular signatures to form the basis of diagnostic assays. The nucleotide signatures associated with CIP-resistance will be useful for diagnostic tests to rapidly identify CIP resistant *B. anthracis* and to infer the level of resistance of these mutant strains. We illustrate the diagnostic potential of these signatures using

a primer extension assay (SNaPshot, Applied Biosystems Incorporated). PCR and extension primers are presented to allow the detection of these signatures.

Mutant	MCI [CIP μg/ml]	gyrA mutation Δ Nucleotide	Δ Amino Acid	parC mutation Δ Nucleotide	Δ Amino Acid
S1-1	0.38	C254 → T	S85 →L		
S1-2	0.38	G265 → A	E89 →K		
S1-3	≥0.25	G248 → A	G83 →D		
S1-4	≥0.25	G250 → A	D84 →N		
S1-5	≥0.25	G247 → T	G83 →C		
S2-1	12	C254 → T	S85 →L	C242 →T	S81 →F
S2-2	4	C254 → T	S85 →L	C242 →A	S81 →Y
S2-3	≥1.5	C254 → T	S85 →L	G253 →A	E85 →K
S2-4	≥1.5	C254 → T	S85 →L	A254 →G	E85 →G
S3-1	64	C254 → T	S85 →L	C242 →T	S81 →F
		G265 → A	E89 →K		
S3-2	64	C254 → T	S85 →L	C242 →T	S81 →F
		A266 → C	E89 →A		
S3-x	16, 24, 48, 64	C254 → T	S85 →L	C242 →T	S81 →F

The invention is further described with reference to Appendix A: *In Vitro* Selection and Characterization of High-Level Ciprofloxacin Resistant Mutants in *Bacillus anthracis* by L. Price, et al., the entire contents of which are hereby incorporated by reference. The SNaPshot primer extension assay is described in Appendices B-D, the contents of which are also hereby incorporated by reference in their entireties.

1 49212/CAB/A599

All patents, patent applications, and other publications mentioned in this specification or the attached appendices are incorporated herein in their entireties by reference.

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WHAT IS CLAIMED IS:

1. A method of detecting the presence or absence of a plurality of selected target sequences associated with fluoroquinolone resistance in Bacillus anthracis, comprising

providing a sample comprising *B. anthracis* target polynucleotide, wherein the target polynucleotide is selected from the group comprising *gyrA* gene, *parC* gene, and mixtures thereof,

adding to the target polynucleotide, a plurality of sequence-specific probes, each comprising an oligonucleotide having a probe-specific sequence of nucleotides designed for base-specific binding of the oligonucleotide to a target sequence and each having a probe-specific number of nucleotides, which imparts to the probe a distinctive elution characteristic in a chromatographic separation medium,

reacting the probes with the target polynucleotide under conditions favoring binding of the probes in a base-specific manner to the target polynucleotide,

treating the probes to selectively modify those probes which have bound to the target polynucleotide in a sequence-specific manner, to form modified, labeled probes, each having a detectable reporter label and a distinctive elution characteristic in a chromatographic separation medium,

fractionating the modified, labeled probe(s) by chromatography in a chromatographic medium, and

detecting the modified, labeled probe(s) to determine the presence or absence of the selected target sequences.

2. The method of claim 1, wherein the sequence-specific probes are selected from the group comprising BA*gyrA*SNP247F_internal, BA*gyrA*SNP248F_internal, BA*gyrA*SNP250F_internal, BA*gyrA*SNP254R_internal,

BA*gyrA*SNP254R(G)_internal, BA*gyrA*SNP254R(T)_internal, BA*gyrA*SNP254R(GT)_internal, BA*gyrA*SNP265R_internal, BA*gyrA*SNP265R(G)_internal, BA*gyrA*SNP266R_internal, BA*parC*SNP242F_internal, BA*parC*SNP253R_internal, BA*parC*SNP254R_internal, and mixtures thereof.

3. The method of claim 1, further comprising the step of amplifying a sample containing *B. anthracis* nucleic acid with a pair of primers selected from the group comprising BA*gyrA*01F_flanking/BA*gyrA*01R_flanking, BA*parC*02F_flanking/BA*parC*02R_flanking and mixtures thereof, to thereby produce the *B. anthracis* target polynucleotide.

4. A purified oligonucleotide that has a nucleotide sequence that is set forth as BA*gyrA*SNP247F_internal.

5. A purified oligonucleotide that has a nucleotide sequence that is set forth as BA*gyrA*SNP248F_internal.

6. A purified oligonucleotide that has a nucleotide sequence that is set forth as BA*gyrA*SNP250F_internal.

7. A purified oligonucleotide that has a nucleotide sequence that is set forth as BA*gyrA*SNP254R_internal.

8. A purified oligonucleotide that has a nucleotide sequence that is set forth as BA*gyrA*SNP254R(G)_internal.

9. A purified oligonucleotide that has a nucleotide sequence that is set forth as BA*gyrA*SNP254R(T)_internal.

10. A purified oligonucleotide that has a nucleotide sequence that is set forth as BA*gyrA*SNP254R(GT)_internal.

11. A purified oligonucleotide that has a nucleotide sequence that is set forth as BA*gyrA*SNP265R_internal.

12. A purified oligonucleotide that has a nucleotide sequence that is set forth as BA*gyrA*SNP265R(G)_internal.

13. A purified oligonucleotide that has a nucleotide sequence that is set forth as BA*gyrA*SNP266R_internal.

14. A purified oligonucleotide that has a nucleotide sequence that is set forth as BA*parC*SNP242F_internal.

15. A purified oligonucleotide that has a nucleotide sequence that is set forth as BA*parC*SNP253R_internal.

16. A purified oligonucleotide that has a nucleotide sequence that is set forth as BA*parC*SNP254R_internal.

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In Vitro Selection and Characterization of High-Level Ciprofloxacin Resistant Mutants in *Bacillus anthracis*

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(Abstract)

In the US, ciprofloxacin is the most commonly prescribed antibiotic for both prophylactic and therapeutic treatment of anthrax. In order to characterize fluoroquinolone resistance in *Bacillus anthracis*, we used an *in vitro* stepwise selection procedure to isolate spontaneous high-level ciprofloxacin (CIP) resistant mutants. In a series of three selection steps, we were able to isolate *B. anthracis* mutants with MICs as high as 64 μg CIP/ml (1000 fold increase over the wild-type MIC). We found that first-level mutants, selected on 0.25 μg CIP/ml, developed 1 of 5 missense point mutations in the Quinolone Resistance Determining Region (QRDR) of the *gyrA* gene. The most common of these mutants (Ser 85 → Leu) was used in a second selection on 1.5 μg CIP/ml. Second-level mutants developed 1 of 4 missense mutations in the QRDR of *parC*. The most common second-step mutant (Ser 81 → Phe) was used in a third and final selection on 24 μg CIP/ml. Two strains isolated from the third step had additional missense point mutations in the *gyrA* QRDR. However, all other third level isolates were devoid of mutations within the four known QRDRs and exhibited no evidence of increased multi-drug efflux. Hence, the genetic basis for these CIP resistant mutants remains unknown. We measured mutation rates using a Luria-Delbruck fluctuation test and found the stepwise phenotypic rates (step 1 = 6.6×10^{-10}; step 2 = 1.0×10^{-8}; and step 3 = 4.8×10^{-10} mutants per generation) to be similar to those reported in other species. These typical spontaneous nucleotide substitution rates support the recent emergence, long dormancy and/or population constriction hypotheses for the lack of genetic diversity among *B. anthracis* strains. The predicted combined mutation rate for a wild-type *B. anthracis* strain to mutate to the highest resistance level in a single step is absurdly low (3.2×10^{-27}). However, the misuse of CIP could defeat this three-step barrier to high-level resistance and argues for the judicious use of this clinically important antibiotic. Finally, we have developed a molecular assay for screening *B. anthracis* for single nucleotide polymorphism (SNPs) associated with CIP resistance. This diagnostic approach allows a large number of isolates to be screened rapidly and in situations where culturing the sample is not possible.

INTRODUCTION

Bacillus anthracis regularly infects livestock and wild ungulates causing the disease anthrax. Although globally dispersed and endemic to many regions, *B. anthracis* shows little genetic variation between strains. Population studies using various methods of analysis including Pulse Field Gel Electrophoresis (PFGE) (Harrell *et al.*, 1995), Single Nucleotide Polymorphisms (SNP) (Price *et al.*, 1999; Harrell *et al.*, 1995), Amplified Fragment Length Polymorphisms (AFLP) (Keim *et al.*, 1997) and Multi-Locus Variable Number Tandem Repeat Analysis (MLVA) (Keim *et al.*, 2000) have all found *B. anthracis* to be highly monomorphic. The paucity of single nucleotide polymorphisms between strains has led to speculation of three hypotheses: (1) It is possible that *B. anthracis* has a reduced per-nucleotide mutation rate. (2) As with many other pathogens, it is possible that *B. anthracis* is a recently emerged pathogen or at least went through a major population restriction recently.(3) As a spore forming bacterium *B. anthracis* may have greatly reduced evolution rates due to extended dormancy periods (Keim *et al.*, 1997; Hugh-Jones, 1999; Keim *et al.*, 2000). These three theories are not mutually exclusive and could together explain the striking level of homology between isolates from around the world. The reduced rate of nucleotide substitution hypothesis is most easily resolved and can be tested by examining mutation rates *in vitro*.

A spore-forming zoonotic, *B. anthracis* occasionally infects humans, causing cutaneous, intestinal or pulmonary forms of anthrax (Friedlander, 1999). Although all three human forms are rare, the potential for using *B. anthracis* as a biological weapon makes development of antibiotic resistance a particularly relevant concern. Currently, the two preferred therapeutics for human anthrax infections are penicillin G and ciprofloxacin ((Friedlander *et al.*, 1993 and Friedlander, 1999). While resistance to penicillin G has been reported in natural isolates and a

genetically engineered vaccine strain of *B. anthracis* (Odendaal *et al.*, 1991; Patra *et al.*, 1998; Pomerantsev *et al.*, 1992), natural fluoroquinolone resistance is undocumented. Recently, however, Choe, *et al.* and Brook, *et al.* reported developing fluoroquinolone resistant strains *in vitro* (Choe *et al.*, 2000; Brook *et al.*, 2001).

Fluoroquinolone bactericidal action is on gyrase-DNA and topoisomerase IV-DNA complexes where drug binding causes the release of double-stranded DNA breaks (Drlica and Zhao, 1997, Piddock, 1999). Fluoroquinolone resistant mutants have amino acid changes in Quinolone Resistance Determining Regions (QRDRs) of the GyrA subunit of gyrase and the ParC subunit of topoisomerase IV. Resistance can also arise from the over-expression of multi-drug efflux pumps of the major facilitator superfamily. Low-level resistance can be acquired with a single missense mutation within a QRDR or a point mutation in the regulatory region of an efflux pump. However, high-level resistance requires a combination of mutations. The stepwise accumulation of QRDR mutations required for high-level resistance appears to follow a species-specific and predictable pathway (Ng *et al.*, 1996; Ferrero *et al.*, 1995).

Fluoroquinolone resistance, like resistance to many other antibiotics, is becoming prevalent in several clinically important species including, *Staphylococcus aureus*, *Campylobacter jejuni* and *Streptococcus pneumoniae* (Ball, 1990; Trucksis *et al.*, 1991; Segreti *et al.*, 1992; Ferrero *et al.*, 1994; Charvalos *et al.*, 1995). While non-compliance with recommended fluoroquinolone regimens is thought to be a major source of new resistant strains, other studies indicate that standard regimens themselves might be insufficient for producing inhibitory concentrations of fluoroquinolones in the soft tissue of patients (Brunner *et al.*, 1999). In addition to clinical sources, the use of fluoroquinolones in food-animal production has been identified as a major contributor to the emergence of fluoroquinolone resistance (Endtz *et al.*,

1990; van den Bogaard *et al.*, 2000; van den Bogaard *et al.*, 2001). The sub-inhibitory concentration used for antimicrobial growth promotion in animal production is thought to pose a particular risk for the development of resistant food-borne pathogens (Schwarz *et al.*, 2001; van den Bogaard, 2000). Further studies using standardized methods (Caprioli *et al.*, 2000) must be performed to determine the relative risk associated with these factors and to determine those microbes most likely to develop resistance to the fluoroquinolones.

In order to study fluoroquinolone resistance and mutation rates in *B. anthracis*, we used an *in vitro* stepwise selection procedure (Ferrero *et al.*, 1995) to develop high-level ciprofloxacin resistant mutants. Mutation rates were determined using a Luria-Delbruck model and genotypes of resistant strains were determined at each step. Fluoroquinolone minimal inhibitory concentrations (MICs) were determined for each unique mutant and evidence for multi-drug efflux was assayed. Single nucleotide changes associated with Cipro-resistant mutants are described along with a rapid assay for detecting such mutants directly from their DNA.

MATERIALS AND METHODS

Bacterial strains. Selections were performed on the non-virulent, pX01-/ pX02-, Ames strain of *B. anthracis* (Ivins *et al.*, 1986). All DNA samples used for the diversity study came from our *B. anthracis* DNA collection (Keim *et al.*, 2000).

Diversity Study. The QRDRs of *gyrA* and *parC* were sequenced from eight major diversity groups and analyzed for point mutations as described below. The eight strains, 3 (74-42C-8), 25 (14185), 39 (46), 45 (2B80), 62 (Oct-32), 77 (Vollum), 80 (RA3) and 87 (K88) were described previously by Keim, *et al.* (Keim *et al.*, 2000).

Note to reviewers : Below are two alternative version of this paper. We are suggesting that the first and abreviated form be published and that we would then provide the second version upon request.

Version 1 :

Stepwise mutant selection. Spontaneous mutants from the *B. anthracis* Ames strain missing both virulence plasmids, were selected using standard microbiological techniques in a stepwise fashion (details available upon request). The most common genotype observed in each step was used for subsequent selections on the next higher cipro step (S1-1 and S2-1, respectively).

Version 2 :

Stepwise mutant selection. *B. anthracis* Ames -/- strain was taken from a frozen stock, streaked onto blood agar plates and grown overnight at 35°C. Cells from isolated colonies were used to inoculate culture tubes containing 5 ml of Mueller-Hinton broth. Cultures were incubated overnight at 37°C in a G24 Environmental Incubator Shaker (New Brunswick Scientific, Edison, NJ, USA) shaking at 225 rpm. Each of these cultures (mean OD_{625} ~1.4 or 1.43 x 10^8 CFU/ml) was transferred to a 0.45 µm nitrocellulose membrane filter (Millipore, Bedford, MA USA). Membranes were placed cell-side-up onto Mueller-Hinton agar containing 0.25 µg CIP/ml and incubated for ~40 h. Cells from a single colony from each positive plate were streaked onto blood agar and grown overnight at 35°C. Cells from these plates were used to prepare frozen stocks and to isolate DNA for sequencing (see below). The most common unique genotype, S1-1, was subjected to a subsequent round of selection on Mueller-Hinton agar containing 1.5 µg CIP/ml. Likewise, the most common genotype from this selection, S2-1, was subjected to a third and final selection on agar containing 24 µg CIP/ml.

Mutation rates. Mutation rates for steps 1, 2 and 3 ciprofloxacin resistant mutants were determined using 96 independent cultures of the wild type Ames -/-, S1-1, and S2-1, respectively. A single colony of the starting isolate was suspended in LB broth and used to inoculate each of the independent cultures with approximately 1,000 cells. For steps 1 and 3 mutants, 96 1 ml cultures were grown in LB broth in four 24-well plates (Costar). For step 2, 96 100 µl were grown in LB broth cultures in a single 96-well plate (Costar). All plates were incubated overnight at 37°C in a G24 Environmental Incubator Shaker (New Brunswick Scientific, Edison, NJ, USA) shaking at 225 rpm. Six cultures were chosen at random for each step and used to determine the average total number of cells present in each culture. The remaining 90 cultures were plated onto Mueller-Hinton ciprofloxacin plates with concentrations of 0.25 µg CIP/ml, 1.5 µg CIP/ml, and 24 µg CIP/ml for steps 1, 2, and 3, respectively. For step 2, the 100 µl cultures were directly plated. For steps 1 and 3, the 1 ml cultures were transferred to sterile 1.5 ml microcentrifuge tubes and centrifuged at 3,000 x g for 5 min. Approximately 850 µl of the supernatant was removed, the pellet was resuspended in the remaining broth and plated. All of the plates were incubated at 37°C for ~48 h. Up to four putative resistant colonies from each positive plate were transferred to fresh selective medium, and incubated at 37°C for ~48 h to confirm resistance. The number of plates devoid of resistant mutants represents zero mutational events. This value was used with the cell count in the Poisson distribution to estimate the mutation rate for each step.

Susceptibility testing. MICs were determined by the Mueller-Hinton agar dilution method according to the guidelines of the National Committee for Clinical Laboratory Standards (National committee, 1997). The E-test strips (AB BIODISK) were used for rapid screening and are shown in Figure 1.

DNA extraction. DNA was extracted as described in Keim *et. al.*, 2000. Briefly, DNA was extracted from each resistant mutant by suspending ~ 1 mg of cellular material from blood agar plates in 150 µl of heat-soak buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0) and heating to 85°C for 30 min. Cellular debris was pelleted by centrifugation and the supernatant was used as template in PCR reactions.

PCR amplification. All primers (Table 1) were designed from the incomplete *B. anthracis* genome sequence generously provided by The Institute for Genomic Research, Rockville, MD, USA. PCR products were amplified in 50 µl PCR reactions and prepared as follows: 1X PCR buffer (20 mM Tris pH 8.4, 50 mM KCl) (Gibco/BRL, Bethesda, MD, USA), 0.10 mM DNTPs, 2 mM $MgCl_2$, 2 µl heat-soak supernatant as template, 0.04 U/µl *Taq* DNA Polymerase (Gibco/BRL, Bethesda, MD, USA), 0.2 µM forward and reverse primers, adjusted to 50 µl with filtered (0.2 µm) 17.8 mOhm E-pure water. Reactions were heated to 94°C for 5 min, then subjected to 35 cycles of 20 s at 94°C, 20 s at 60°C and 20 s at 72°C. This was followed by heating to 72°C for 5 min to complete primer extension. PCR products were quantified on EtBr stained 1.5% SyncrgcI™ (Diversified Biotech, Boston, MA, USA)/0.7% agarose (Gibco/BRL, Bethesda, MD, USA). Quantified PCR products were sequenced as described below.

DNA sequencing. PCR products were diluted 1:5 in water and sequenced on an ABI377 fluorescent sequencer using the ABI PRISM® Ready Reaction BigDye™ Terminator Cycle Sequencing Kit (both from Perkin-Elmer/Applied Biosystems Inc., Foster City, CA, USA). When necessary, contiguous gene sequences were prepared from the individual sequences using SeqMan™ software (DNASTAR, inc., Madison, WI, USA). Contiguous Sequences were aligned with the wild-type sequences using MegAlign™ software (DNASTAR, inc., Madison, WI, USA).

SNP assay. A single nucleotide polymorphism (SNP) assay was developed to rapidly identify the nine observed resistance mutations (Table 2). Flanking primers (Table 3) were designed to amplify bases 203-323 of *gyrA* (122 bp product) and bases 175-320 of *parC* (146 bp product). PCR products were amplified in 10 µl singleplex or duplex PCRs with final concentrations of 1x PCR Buffer (above), 3mM $MgCl_2$, 0.1 mM dNTPs, forward and reverse primer pairs (0.1 µM *gyrA* primers / 0.4 µM *parC* primers), 1 U Platinum® *Taq* DNA Polymerase (Gibco/BRL, Bethesda, MD, USA), and 1 µl heat-soak supernatant as template. Reactions were heated to 94°C for 5 min, then subjected to 30 cycles of 20 s at 94°C, 30 s at 60°C and 30 s at 72°C. The remainder of the procedure was carried out according to instructions in the ABI PRISM® SNaPshot™ Multiplex Kit and run on an AB3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Single base extension (SBE) primers (Table 3) were designed with polynucleotide tails (poly-Cs and single As) to customize amplicon lengths to 4-bp intervals such that when separated electrophoretically, the six *gyrA* SNPs were detected in the 5' to 3' order in which mutations are found, followed by the three *parC* SNPs (also in 5' to 3' order of occurrence). Since primers *gyrA*265 and *gyrA*254 overlapped 1 and 2 SNP loci respectively, they were designed with degenerate base pairs at sites 266 and 265 to allow annealing on templates with step 3 mutations. Despite primer degeneracy, amplification of *gyrA*265 in the 13-primer multiplex was weak on S3-2 mutants. This locus was therefore targeted individually by performing a second SBE containing only the two *gyrA*265 primers when a template was suspected to be an S3-2 mutant. The order of SBE products enabled multiplexing of SBE PCRs and facilitated scoring, eliminating the need for a size standard. Therefore, this assay can be performed on a 4-dye ABI377 if a 5-dye capillary machine is not available.

RESULTS AND DISCUSSION

The sequential selection of *B. anthracis* on increasing CIP concentrations resulted in a stepwise accumulation of mutations, leading to the isolation of mutant strains with MICs as high as 64μg CIP/ml (1000-fold higher than wild-type) (Table 2). The accumulation of mutations occurred in a distinctive and ordered manner. First level mutants, selected on 0.25 μg CIP/ml, developed at a rate of 6.6×10^{-10} and had one of five mutations within the *gyrA* QRDR (Table 2). A disproportionate number (71%) of these mutants possessed the C254→T missense mutation in *gyrA* (Table 2). The level of resistance conferred by this mutation was similar to that of other S1 mutations. Since this mutation provided no selective advantage over the other S1 mutations, it is reasonable to call the C254 nucleotide of *B. anthracis gyrA* a mutational hotspot. Second level mutants, selected on 1.5 μg CIP/ml, developed at a rate of 1.0×10^{-8} and possessed one of four mutations within the *parC* QRDR (Table 2). As with the S1 mutants one S2 genotype, C242→T, was overrepresented (71%) (Table 2). While it is likely that the C242 nucleotide of *parC* represents another mutational hotspot, the overrepresentation could also be a result of the disproportionate level of resistance conferred to the strain by the mutation (Table 2). Third level mutants, selected on 24 μg CIP/ml, developed at a rate of 4.8×10^{-10}. Two-third-level mutants were identified with novel mutations within the *gyrA* QRDR (Table 2). However, the other 21 third-level mutants had no additional alterations in either the *gyrA* or *parC* QRDRs. Potential QRDRs in *gyrB* and *parE* were also sequenced from these strains and revealed no additional mutations in these regions. The targeted stepwise accumulation of mutations (S1 *gyrA* → S2 *parC* → S3 *gyrA*/?) give further evidence to support the hypothesis that particular fluoroquinolones have different primary topoisomerase targets within various bacterial species (Ferrero *et al.*, 1995; Ng *et al.*, 1996; Pan and Fisher *et al.*, 1998). These data indicate that the

primary target of CIP in wild-type *B. anthracis* is GyrA, the secondary target is ParC and the tertiary targets are yet to be fully determined. The target order of CIP appears to be determined by the amino acid residues of the Gyrase and Toposiomerase IV subunit QRDRs. Hence, the results of the QRDR diversity study presented in this paper suggest that this order of targeting will hold true for most *B. anthracis* isolates.

The absence of additional missense mutations in either the *gyrA* or the *parC* QRDRs of most (92%) third-step mutants was perplexing. Blast™ Searches of the partial *B. anthracis* genome sequence with the gene sequences of the *B. cereus* and *B. subtilis* multi-drug efflux pumps, *bmr* (gb L25604) and *blt* (gb L32599) (Ahmed *et al.*, 1995) revealed regions of high homology. As members of the major facilitator superfamily, both of these pumps have been shown to externalize a diverse group of chemicals, including flouroquinolones, ethidium bromide, rhodamine dyes, and chloramphenicol (Neyfakh, 1992; Neyfakh *et al.*, 1993; Ahmed *et al.*, 1995). However, MICs of ethidium bromide were not increased in any of the CIP resistant mutants and, therefore, not indicative of an up-regulation of such membrane pumps (data not shown). Notably, Blast™ searches with the regulatory sequences of *bmr* and *blt*, *bmrR* (gb L25604) and *bltR* (gb L32599), respectively, revealed no significant homology within the *B. anthracis* genome (Ahmed *et al.*, 1995). The nature of resistance in most CIP resistant bacterial strains has been attributed to either an amino acid change in one of the four topoisomerase genes or to the up-regulation of a multi-drug efflux pump (Drlica and Zhao, 1997; Piddock, 1999). However, a novel method of fluoroquinolone resistance was described recently, which was facilitated by the plamid-encoded Qnr protein.The 218-aa Qnr protein belongs to the pentapeptide repeat family and functions by protecting DNA gyrase from inhibition by fluoroquinolones (Tran and Jacoby, 2002). Therefore, one cannot discount the possibility that the

third-level *B. anthracis* mutants, devoid of additional point mutations in the QRDRs, have developed a novel pathway to protection. However, investigation of this possibility was beyond the scope of this study.

This study demonstrates the ability of *B. anthracis* to develop a number of different missense mutations that enable it to grow in the presence of CIP. The stepwise phenotypic rates at which *B. anthracis* develops resistance to CIP (4.8×10^{-10} to 1.0×10^{-8}) are similar to those reported for fluoroquinolone resistance in other species (Pan and Fisher, 1998; Schedletzky *et al.*, 1999). The rarity of human anthrax cases and the carcass-dependent transmission cycle of this pathogen make the development and spread of CIP resistant *B. anthracis* through patient non-compliance unlikely. However, the agricultural practice of antimicrobial growth promotion does have this potential outcome. CIP regimens targeted at serum and tissue concentrations of ≥0.38 μg CIP/ml would reduce the chances for developing CIP resistant *B. anthracis* by requiring the statistically unlikely event (6.6×10^{-18}) of a bacterium to develop advantageous mutations in the *gyrA* and *parC* QRDRs simultaneously. As such, this concentration (0.38 μg CIP/ml) appears to meet what Dong *et al* (1999) have termed the Mutant Prevention Concentration (MPC) for *B. anthracis*. The serum and tissue concentrations resulting from the low-level feeding of antibiotics as antimicrobial growth promoters in livestock would not reach the MPC and likely fall short of the MIC for wild-type *B. anthracis*. Therefore, this practice could present a potential risk for the development of resistant strains, particularly in those livestock regions to which *B. anthracis* is endemic.

Should strains of CIP resistant *B. anthracis* arise, either by misuse of antibiotics or malevolence, they can be rapidly genotyped using the described SNP assay (Table 3, Figure 2). This assay was developed using the SNaPshot™ technology described in the Materials and

Methods, but many other SNP assays are available. In this assay, the mutational status of the six *gyrA* and three *parC* nucleotides is easily observed in a single lane on an ABI377 or AB3100, since all thirteen SBE primers may be multiplexed to amplify nine loci (Table 3, Figure 2). As discussed above, not all mutations responsible for CIP resistance were identified in this study. However, the most common ones (found in step 1 and 2 mutants) were readily assayed using the SNP assay. Because these nine mutations play a critically important role in determining the level of CIP resistance, SNP information would be useful in developing an appropriate antibiotic treatment strategy at an early stage of an outbreak. A newly acquired strain could be genotyped in just a few hours. There are many more SNP assay platforms available and these nucleotide changes are readily adaptable to the most appropriate technology.

In order to determine the level of natural variation within the QRDRs of *gyrA* and *parC*, we sequenced these genes from a set of *B. anthracis* isolates representing the eight major diversity groups within the species (Keim et al., 2000). Not surprisingly, the survey revealed no sequence differences between these phylogenetically distant *B. anthracis* strains. Despite the overall lack of genetic diversity between *B. anthracis* strains, we did not find the per-gene mutation rates in *gyrA* and *parC* to be substantially lower than in other species. Indeed, the rifampicin mutation rates in Bacillus anthracis are comparable to those reported here (Vogler et al. 2001). If the mutation rates had been substantially lower than those of other bacteria, one could have hypothesized that a species-specific reduced mutation rate (e.g. through enhanced replication or repair capabilities) was involved in the low genetic diversity among *B. anthracis* isolates. Instead, the results of this study, demonstrating a normal rate of mutation, indicate that the recent emergence/population constriction or reduced evolutionary rates due to extended

dormancy hypotheses more likely account for the lack of genetic diversity among *B. anthracis* isolates.

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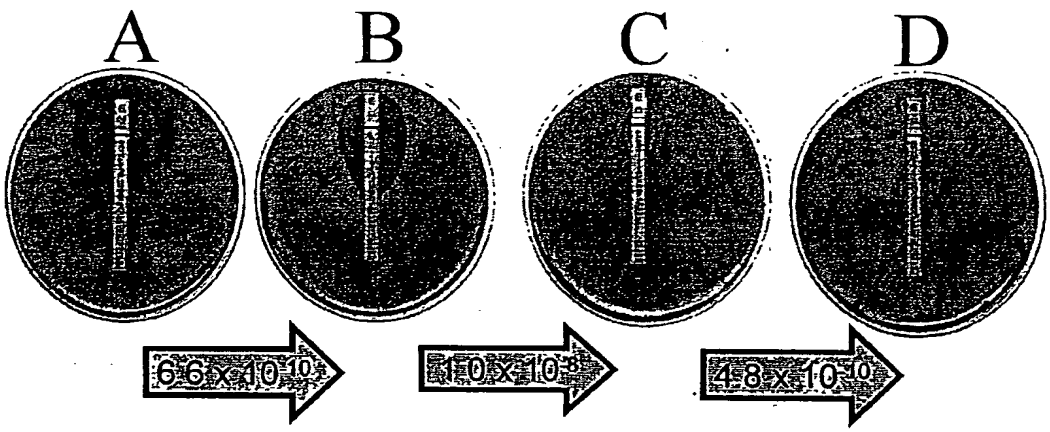
Figure Legends

Figure 1. Mutant Sensitivity to Different Ciprofloxacin Concentrations. The wild type progenitor strain (A) and three sequential step-wise mutant strains (B-D) are evaluated for ciprofloxacin sensitivity using E-strips. The stepwise mutants are (B) S1-1, (C) S2-3 and (D) S3-1. The spontaneous stepwise mutation rates in changes per generation shown in the arrows.

Figure 2. SNP assays for mutational changes associated with CIP resistance. An illustration of an ABI377 gel image is shown with nine SNP loci across seven *B. anthracis* strains (wild type, two step 1 mutants, two step 2 mutants, and two step 3 mutants). In addition, one electropherogram of the wild type genotype generated on a capillary electrophoresis instrument (AB3100) is shown to illustrate the assay's flexibility across diagnostic platforms. SNP of mutant genotypes are: S1-1: *gyrA*254(R) C→T; S1-2: *gyrA*265(R) G→A; S2-1: *gyrA*254(R) C→T & *parC*242 C→T; S2-2: *gyrA*254(R) C→T & *parC*242 C→A; S3-1: *gyrA*254(R) C→T & *gyrA*265(R) G→A & *parC*242 C→T; S3-2: *gyrA*254(R) C→T & *gyrA*266(R) A→C & *parC*242 C→T.


A
B
C
D
6.6 x 10-10
1.0 x 10-8
4.8 x 10-10

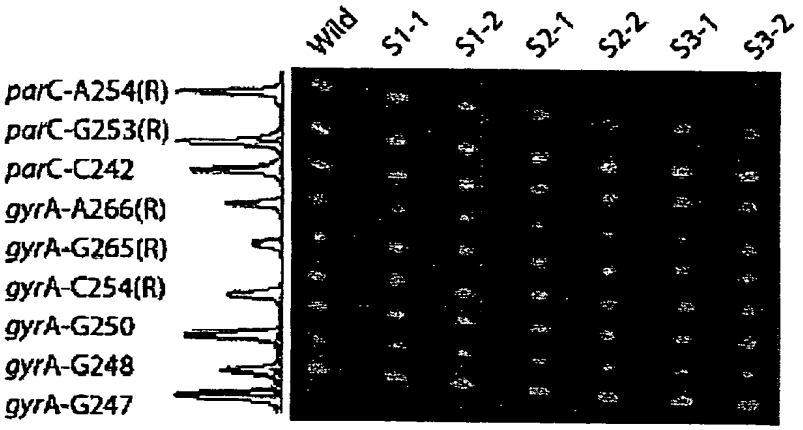


Figure 2: Illustration of ABI377 gel image showing Wild Type genotype and SNP mutations for 2 possible genotypes from each of 3 steps (S1-1: *gyrA*-C254(R)→T; S1-2: *gyrA*-G265(R)→A; S2-1: *gyrA*-C254(R)→T & *parC*-C242→T; S2-2: *gyrA*-C254(R)→T & *parC*-C242→A; S3-1: *gyrA*-C254(R)→T & *gyrA*-G265(R)→A & *parC*-C242→T; S3-2: *gyrA*-C254(R)→T & *gyrA*-A266(R)→C & *parC*-C242→T) and electropherogram of wild type genotype run on ABI3100 with Liz 120 Size Standard (standard not shown).

Table 1. Primers used in this study.

Name	Sequence (5'→3')	Gene/Region
ParC QRDR F	GTGTTAGGTGACCGCTTTGCACGTTATAGTAAATA	*parC*/QRDR
ParC QRDR R	GTAAAACAACCGGTTCTTCACTCGTATCATC	*parC*/QRDR
GyrA QRDR F	ACGTATTAATTCCATAGAGATTTTAGACATTCTTGCTTCTGTATA	*gyrA*/QRDR
GyrA QRDR R	CATTTTTAGATTACGCAATGAGTGTTATCGTATCTCG	*gyrA*/QRDR
BA ParC aF1	GGTACGACAGTTGCCCAAAATGATGGTT	*parC*
BA ParC aR1	CAAGCGGAAGCAATTGTATCCT	*parC*
BA ParC bF1	CGCGTCGATCATCACTATATGTTTTCTTAACTCTC	*parC*
BA ParC bR1	ATTATTATTCGCGGGAAAGCAGAGGTTGA	*parC*
BA ParC cF1	GTCTCATCACGTACTTCAGCAATGCCATCT	*parC*
BA ParC cR1	TCGGCTAAAACAGTCGGTAACGTTATTGGTAA	*parC*
BA ParC-E F1	CGGATCCCCGTCAACAC	*parC* & *parE*
BA ParC-E R1	CGGATCAATTATGGGAAACAACGATGAATC	*parC* & *parE*
BA ParE aF1	AAGCGGGAGGTCATGAAACTTCTCTGC	*parE*
BA ParE aR1	AGTGGTAAGTTAACACCCGCACAATCACG	*parE*
BA ParE bF1	CCCTTGTTTCGCAGAACCAC	*parE*
BA ParE bR1	TTGAAGCTTTCGTTTCCTAT	*parE*
BA ParE cF1	CTAATTCTGCTTCAATCCCATTTTGTTCACC	*parE*
BA ParE cR1	TAGCGTTATAGATAAAGGGCGAGGAATG	*parE*
BA ParE dF1	ACACCGCCATTTTCAAAGCGTTGTTC	*parE*
BA ParE dF1	ACACCGCCATTTTCAAAGCGTTGTTC	*parE*
BA ParE dR1	GATTTTGGATTAGGAAAGGGGCAAGGAGTT	*parE*
BA GyrB aF1	CGACGGAATTGAACACGAAACA	*gyrB*
BA GyrB aR1	TACAGATGCCCCAACACC	*gyrB*
BA GyrB bF1	ATGGGACGTCCTGCTGTAGAAGTTATTATGACC	*gyrB*
BA GyrB bR1	AGTTAAACCTTCACGAACGTCCTCACCAGTTA	*gyrB*
BA GyrB cF1	ACGTATGAAGGTGGAACACATGAAGTAGGGTTTA	*gyrB*
BA GyrB cR1	GCTTTCTCAATATCAAAATCTCCGCCAATGT	*gyrB*
BA GyrB dF1	CGTCACTTCCAAGCGATTTTACCACTGAA	*gyrB*
BA GyrB dR1	ACCTCCTCTTACATTTCCGTTACACATACATTGATTTAT	*gyrB*
BA GyrB-A F1	GGGGGATAAAGTAGAGCCACGTCGTAACT	*gyrB* & *gyrA*
BA GyrB-A R1	AGGAAAACGCGCTGGTAACA	*gyrB* & *gyrA*
BA GyrA aF1	CAGCAATGCGTTATACAGAAGCAAGAATGTC	*gyrA*
BA GyrA aR1	TGCCTTTTCAAGTTCATAAGCAGTA	*gyrA*
BA GyrA bF1	GGAAGTACGTCGTGATGCCAATGCTAATG	*gyrA*
BA GyrA bR1	ATACCTTTCGCTGTACGACTATACTCTGGGATTTC	*gyrA*
BA GyrA cF1	CAGAACAAAACATCGCCATTACGTTAACTCATAA	*gyrA*
BA GyrA cR1	AGAGATTTGATCAACTGGCATACGAATAATAACACC	*gyrA*

Table 2. Mutant Genotypes Identified in this Study

Mutant	MIC [CIP μg/ml]	Mutation Rate	Frequency (%)	*gyrA* mutation		*parC* mutation	
				Δ Nucleotide	Δ Amino Acid	Δ Nucleotide	Δ Amino Acid
S1-1	0.38	4.7×10^{-10}	71	C254→T	S85→L	-	-
S1-2	0.38	9.4×10^{-11}	3	G265→A	E89→K	-	-
S1-3	≥0.25	3.1×10^{-11}	1	G248→A	G83→D	-	-
S1-4	≥0.25	3.1×10^{-11}	1	G250→A	D84→N	-	-
S1-5	≥0.25	3.1×10^{-11}	1	G247→T	G83→C	-	-
S2-1	12	7.1×10^{-9}	71	C254→T	S85→L	C242→T	S81→F
S2-2	4	1.6×10^{-9}	16	C254→T	S85→L	C242→A	S81→Y
S2-3	≥1.5	1.0×10^{-9}	11	C254→T	S85→L	G253→A	E85→K
S2-4	≥1.5	2.6×10^{-9}	2	C254→T	S85→L	A254→G	E85→G
S3-1	64	2.1×10^{-11}	4	C254→T G265→A	S85→L E89→K	C242→T	S81→F
S3-2	64	2.1×10^{-11}	4	C254→T A266→C	S85→L E89→A	C242→T	S81→F
S3-x	16,24,48,64	?	92	C254→T	S85→L	C242→T	S81→F

Table 3. External and SBE primers used in the SNP assay.

Name	Sequence (5'→3')	Product Size	SNP
External primers			
BA*gyrA*01F_flanking	TCAGCACGTATTGTTGGTGAAG	122	-
BA*gyrA*01R_flanking	TGCCCATCAACAAGCATATAAC	122	-
BA*parC*02F_flanking	AAAGCGTTCCGTAAGTCGG	145	-
BA*parC*02R_flanking	TTATTACCATGCATCTCAACTAAAAC	145	-
SBE primers			
BA*gyrA*SNP247F_internal	ATCGGTAAGTATCACCCTCAT	22	G/T
BA*gyrA*SNP248F_internal	cccccCGGTAAGTATCACCCTCATG	26	G/A
BA*gyrA*SNP250F_internal	ccccccccaGGTAAGTATCACCCTCATGGT	30	G/A
BA*gyrA*SNP254R_internal	ccccccccccccccCCATCGTTTCATAAACAGCT	34	C/T†
BA*gyrA*SNP254R(G)_internal	ccccccccccccccCCATCGTTGCATAAACAGCT	34	C/T†
BA*gyrA*SNP254R(T)_internal	ccccccccccccccCCATCGTTTTATAAACAGCT	34	C/T†
BA*gyrA*SNP254R(GT)_internal	ccccccccccccccCCATCGTTGTATAAACAGCT	34	C/T†
BA*gyrA*SNP265R_internal	ccccccccccccccccccaGCCATACGTACCATCGTTT	38	G/A†
BA*gyrA*SNP265R(G)_internal	ccccccccccccccccccaGCCATACGTACCATCGTTG	38	G/A†
BA*gyrA*SNP266R_internal	cccccccccccccccccccccccCGCCATACGTACCATCGTT	42	A/C†
BA*parC*SNP242F_internal	cccccccccccccccccccccccccccccccCACCCGCACGGTGATT	47	C/T/A
BA*parC*SNP253R_internal	ccccccccccccccccccccccccccccccGACTTAAACGTACCATCGCTT	51	G/A†
BA*parC*SNP254R_internal	ccccccccccccccccccccccccccccccccccAGCGATGGTACGTTTAAGTC	54	A/G†

†SNPs will be detected as reverse complements in the SNaPshot™ assay when reverse SBE primers are used.

Table 2. Mutant Genotypes Identified in this Study

Mutant	MIC [CIP μg/ml]	Mutation Rate	Frequency (%)	*gyrA* mutation		*parC* mutation	
				Δ Nucleotide	Δ Amino Acid	Δ Nucleotide	Δ Amino Acid
S1-1	0.38	4.7×10^{-10}	15/21	C254→T	S85→L	-	-
S1-2	0.38	9.4×10^{-11}	3/21	G265→A	E89→K	-	-
S1-3	≥0.25	3.1×10^{-11}	1/21	G248→A	G83→D	-	-
S1-4	≥0.25	3.1×10^{-11}	1/21	G250→A	D84→N	-	-
S1-5	≥0.25	3.1×10^{-11}	1/21	G247→T	G83→C	-	-
S2-1	12	7.1×10^{-9}	27/38	C254→T	S85→L	C242→T	S81→F
S2-2	4	1.6×10^{-9}	6/38	C254→T	S85→L	C242→A	S81→Y
S2-3	≥1.5	1.0×10^{-9}	4/38	C254→T	S85→L	G253→A	E85→K
S2-4	≥1.5	2.6×10^{-9}	1/38	C254→T	S85→L	A254→G	E85→G
S3-1	64	2.1×10^{-11}	1/23	C254→T G265→A	S85→L E89→K	C242→T	S81→F
S3-2	64	2.1×10^{-11}	1/23	C254→T A266→C	S85→L E89→A	C242→T	S81→F
S3-x	16,24,48,64	?	21/23	C254→T	S85→L	C242→T	S81→F

ABI PRISM®
SNaPshot™ Multiplex Kit

Protocol

For Research Use Only.
Not for use in diagnostic procedures.

Applied Biosystems

APPENDIX B

For Research Use Only. Not for use in diagnostic procedures.

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Contents

Product and Protocol Overview

About the Kit

The ABI PRISM® SNaPshot™ Multiplex Kit is designed to interrogate up to ten single nucleotide polymorphisms (SNPs) at known locations on one to ten DNA templates in a single tube.

Control Template DNA and Primer Mix included in the SNaPshot Multiplex Kit provides reagents for control reactions.

Product Overview

Single nucleotide polymorphisms detection using the ABI PRISM SNaPshot Multiplex Kit requires the following components:

- SNaPshot Multiplex Ready Reaction Mix
- Your template and primers

The ABI PRISM SNaPshot Multiplex Control Template DNA and Primer Mix provides Control Primers and Control Template to perform control reactions only.

Kit Chemistry Based on Single Base Extension

The chemistry is based on the dideoxy single-base extension of an unlabeled oligonucleotide primer (or primers).

- Each primer binds to a complementary template in the presence of fluorescently labeled ddNTPs and AmpliTaq® DNA Polymerase, FS.
- The polymerase extends the primer by one nucleotide, adding a single ddNTP to its 3′ end.

Dye Assignments

The fluorescent dyes are assigned to the individual ddNTPs as follows:

ddNTP	Dye Label	Color of Analyzed Data
A	dR6G	Green
C	dTAMRA™	Black
G	dR110	Blue
T (U)	dROX™	Red

Platforms and Software

Products generated using the ABI PRISM SNaPshot Multiplex Kit can be analyzed with GeneScan® Analysis Software version 3.1 or higher. The kits can be run on the following platforms:

- ABI PRISM® 310 Genetic Analyzer
- ABI PRISM® 3100 Genetic Analyzer
- ABI PRISM® 3700 DNA Analyzer

About This Protocol

This protocol describes how to:

- Prepare sample reactions using your own template(s) and primer(s) or the control template and control primers.
- Perform SNaPshot reactions by thermal cycling and conduct post-extension treatment of the products.
- Electrophorese the samples and analyze the data.

To view a flowchart of the procedure refer to "Overview of the Procedure" on page 9.

Kit Contents and Storage

SNaPshot Multiplex Kit

The ABI PRISM SNaPshot Multiplex Kit is available in three reaction sizes. Using this kit, you can perform your own reactions and also perform 30 control reactions with the control template and primers provided.

One Kit Available in Three Formats

Kit	Number of Reactions[a]	Part Number
ABI PRISM SNaPshot™ Multiplex Kit	100	4323151
	1000	4323154
	5000	4323155

a. Each kit contains Multiplex Control Template and Multiplex Control Primers for 30 control reactions

The kit contains the following items:

Kit Components	Contents
SNaPshot Multiplex Ready Reaction Mix	AmpliTaq® DNA Polymerase, FS
	Fluorescently labeled ddNTPs
	Reaction buffer
SNaPshot Multiplex Control Primer Mix (30 µL total)	20A primer (0.05 pmol/µL)
	28G/A primer (0.10 pmol/µL)
	36G primer (0.05 pmol/µL)
	44T primer (0.30 pmol/µL)
	52C/T primer (0.30 pmol/µL)
	60C primer (0.30 pmol/µL)
SNaPshot Multiplex Control Template (60 µL total)	Amplicon from CEPH DNA
Protocol	P/N 4323357
Quick Reference Card	P/N 4323975

Storing the Reagents

Upon receipt, store the ABI PRISM SNaPshot Multiplex Kit at –15 to –25 °C in a constant-temperature freezer.

Required Software and Materials

Overview

This section describes the software and materials necessary for using the ABI PRISM SNaPshot Multiplex Kit.

GeneScan-120 LIZ Size Standard Recommended

Primers used in a single reaction for multiloci interrogation need to differ significantly in length to avoid overlap between the final SNaPshot products. To analyze the final products successfully and robustly, a 5th dye-labeled internal size standard specifically designed for small fragments should be used. The GeneScan™-120 LIZ™ size standard has been designed specifically for use with the SNaPshot Muiltiplex Kit.

Data Collection Software and/or GeneScan Run Module Required

One of the following Data Collection Software and/or GeneScan Run Modules is required:

310 Genetic Analyzer:

- 310 Data Collection version 2.1
- GS STR POP4 (1 mL) E5

3100 Genetic Analyzer:

- 3100 Data Collection version 1.0
- SNP36_POP4 default module

3700 DNA Analyzer:

- 3700 Data Collection version 1.1 (enabled with 3700 Data Collection 5-Dye Update File P/N 4324208)
- SNP1_1POP5

Materials Required but Not Included

The following materials are required but not included:

Item	Source
One of the following instruments with 5-dye capability: ♦ ABI PRISM 310 Genetic Analyzer ♦ ABI PRISM 3100 Genetic Analyzer with POP-4 polymer and 36-cm array ♦ ABI PRISM 3700 DNA Analyzers with POP-5 polymer and 50-cm array.	Applied Biosystems
GeneAmp® PCR System 9600 thermal cycler with appropriate tubes or plate, and caps	
GeneScan® software v. 3.1 or higher	
Matrix Standard Set DS-02 [dR110, dRGG, dTAMRA™, dROX™, LIZ™]	Applied Biosystems
310	4323050
3100	4323014
3700	4323785
GeneScan™-120 LIZ™ size standard	4324211
Hi-Di™ formamide, 25-mL bottle	Applied Biosystems (P/N 4311320)
1X TE, pH 7.0	Major laboratory supplier (MLS)
Centrifuge with 96-well plate adapter	MLS
Deionized water	MLS
Disposable gloves	MLS
Pipette tips, aerosol resistant	MLS
Shrimp Alkaline Phosphatase (SAP) or	USB Corporation (P/N 70092X, 5000 Units) (P/N 70092Z, 1000 Units) (P/N 70092Y, 500 Units)
Calf Intestinal Phosphatase (CIP)	New England BioLabs (P/N 290L, 5000 Units) (P/N 290S, 1000 Units)

The following materials are required but not included: *(continued)*

Item	Source
Exo I	USB Corporation
or	(Exonuclease I, P/N 70073Z)
PCR Clean Up Kit	Roche Molecular Biochemicals (P/N 1696513, 100 reactions)
or	
High Pure™ PCR Product Purification Kit	Roche Molecular Biochemicals (P/N 1732668, 50 reactions) (P/N 1732676, 250 reactions)

Safety

Documentation User Attention Words

Five user attention words appear in the text of all Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below.

Note Calls attention to useful information.

IMPORTANT Indicates information that is necessary for proper instrument operation.

⚠ CAUTION Indicates a potentially hazardous situation which, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

⚠ WARNING Indicates a potentially hazardous situation which, if not avoided, could result in death or serious injury.

⚠ DANGER Indicates an imminently hazardous situation which, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

Chemical Hazard Warning

⚠ WARNING CHEMICAL HAZARD. Some of the chemicals used with Applied Biosystems instruments and protocols are potentially hazardous and can cause injury, illness, or death.

- Read and understand the material safety data sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with and inhalation of chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.*, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Do not leave chemical containers open. Use only with adequate ventilation.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

Site Preparation and Safety Guide

A site preparation and safety guide is a separate document sent to all customers who have purchased an Applied Biosystems instrument. Refer to the guide written for your instrument for information on site preparation, instrument safety, chemical safety, and waste profiles.

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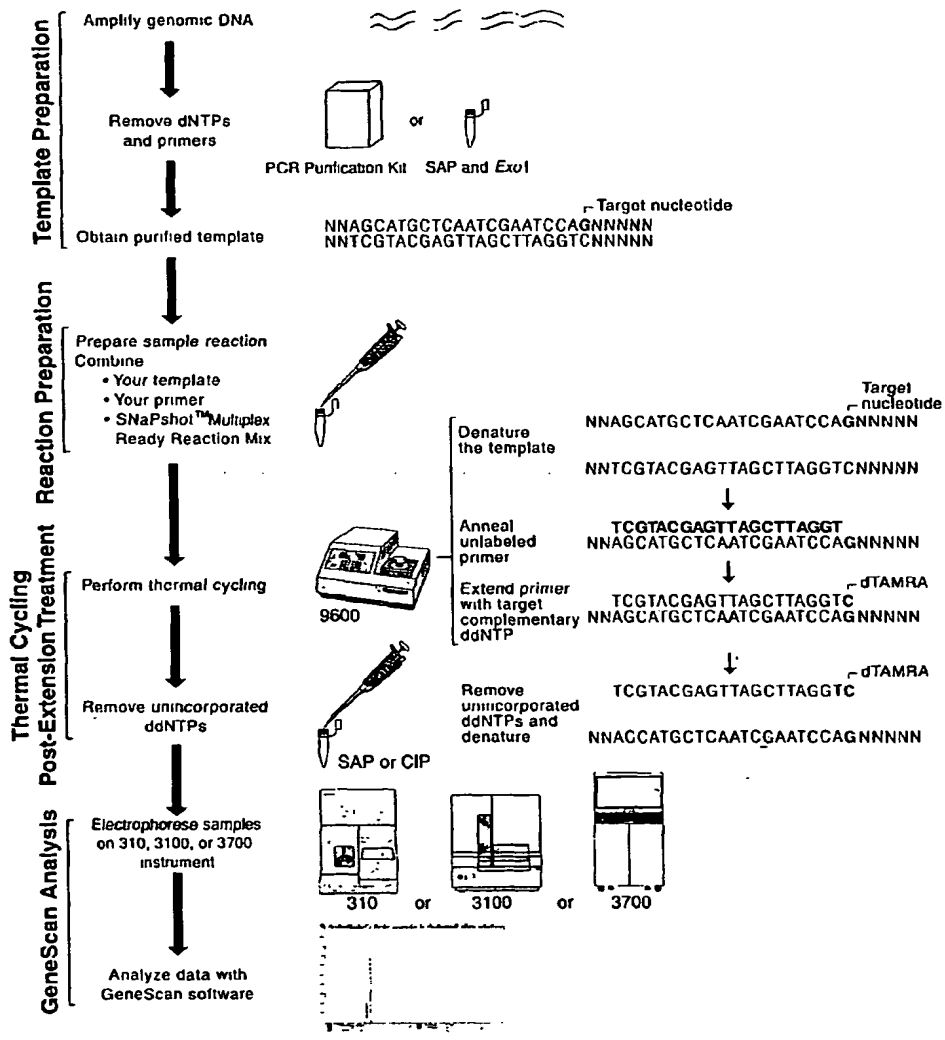
By automated telephone service	Use "Documents on Demand" under "Technical Support."
By telephone in the United States	Dial 1-800-327-3002, then press 1.
By telephone from Canada	**To order in...** / **Dial 1-800-668-6913 and...** English → Press **1**, then **2**, then **1** again French → Press **2**, then **2**, then **1**
By telephone from any other country	See "Regional Offices Sales and Service" under "Technical Support."

Overview of the Procedure



Preparing Your PCR Template for Primer Extension

Purpose

This section describes how to prepare your PCR product template before primer extension.

About the Templates

There are two kinds of templates that you can use in primer extension reactions:

- Plasmid templates
- PCR products

While plasmid templates do not require cleanup before primer extension, PCR product templates must be purified.

Depending on the specific template, 0.01 to 0.40 pmol of the template should be used in the SNaPshot reactions.

Methods for Preparing PCR Templates

After PCR amplification, the resulting template is in solution, along with primers, dNTPs, and enzyme and buffer components. To avoid participation in the subsequent primer-extension reaction, primers and unincorporated dNTPs must be removed.

We recommend the following methods for purifying PCR products:

PCR Purification Kits

High Pure™ PCR Product Purification (P/N 1732668, 50 reactions, 1732676, 250 reactions) or PCR Clean Up Kits (P/N 1696513, 100 reactions) can be purchased from Roche Molecular Biochemicals. Refer to the manufacturer's instructions for the procedure.

SAP and *Exo* I Treatment

To treat PCR products using SAP and *Exo* I:

Step	Action
1	Add the following to 15 µL of PCR product: ♦ 5 units of SAP ♦ 2 unit of *Exo* I Use the following guidelines for enzyme treatment: ♦ Reaction volume can be adjusted up or down. PCR products can be from a single PCR reaction or multiple PCR reactions. We recommend that you purify individual PCR products and combine the purified products in the next step. ♦ **To ensure a low background**, we strongly recommend that the relative ratio of PCR product, SAP, and *Exo* I be kept constant, *i.e.*, 5 units of SAP and 2 units of *Exo* I for every 15 µL of PCR product. ♦ Because of the high glycerol concentration in undiluted SAP and *Exo* I, add each enzyme into the PCR mixture one at a time. ♦ *Exo* I can be freshly diluted in a buffer containing 80 mM Tris-HCl (pH = 9.05) and 2 mM $MgCl_2$. Do not store diluted *Exo* I.
2	Mix thoroughly and incubate at 37 °C for 1 hour. **Note** Because of the high glycerol concentration in undiluted SAP and *Exo* I, vortex briefly to mix.
3	Incubate at 75 °C for 15 minutes to inactivate the enzymes.
4	Keep on ice or at 4 °C. For longer storage, store at –20 °C.

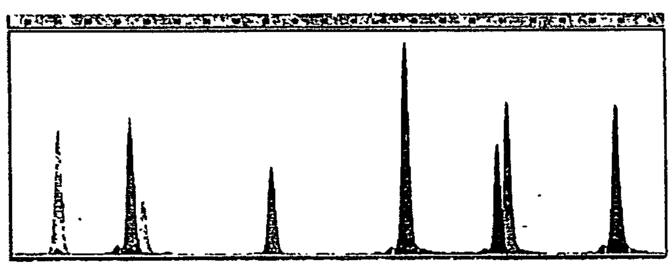
Preparing the Control Reactions

About the Control Reactions Included in each kit are a Multiplex Control Primer Mix tube containing six distinct primers and a Multiplex Control Template tube containing an amplicon from CEPH DNA.

The control primers are listed in the table below.

Multiplex Control Primer Mix	Length of Final Products (nt)	Signal Color	Heterozygosity
20A primer	21	Green	Homozygote
28G/A primer	29	Blue/green	Heterozygote
36G primer	37	Blue	Homozygote
44T primer	45	Red	Homozygote
52C/T primer	53	Black/red	Heterozygote
60C primer	61	Black	Homozygote

Note Due to the influence of the dye on the mobility shift of the DNA fragments, the reported sizes will differ by a few bases from the actual sizes. This is particularly true with the shorter fragments as the relative contribution of the dye is greater.



Primer	20A	28G/A	36G	44T	52C/T	60C
Relative size of SNaPshot product*	24	29	39	46	53	62

*:As determined by sizing against GeneScan-120 Liz size standard

Negative Control Reaction Run one negative control reaction without control template DNA.

Preparing the Control Reactions

To prepare the control reactions:

Step	Action
1	Label two 0.2-mL MicroAmp® tubes, one for the positive control reaction and one for the negative control reaction.
2	Thaw the SNaPshot Multiplex Ready Reaction Mix, Control Template, and Control Reaction Primer Mix **on ice**. Prepare the following reaction mix on ice:

Item	Positive Control (µL)	Negative Control (µL)
SNaPshot Multiplex Ready Reaction Mix	5	5
SNaPshot Multiplex Control Template	2	0
SNaPshot Multiplex Control Primer Mix	1	1
Deionized water	2	4
Total	10	10

3	Mix and spin briefly.
	Note Keep the SNaPshot mixture on ice before putting it into the thermal cycler. **Leaving the mixture at ambient temperature for 20 minutes or longer may result in a higher background.**
4	Proceed to "Thermal Cycling and Post-Extension Treatment" on page 16.

Preparing Your Sample Reactions

Overview This section describes how you set up multiplex SNaPshot reactions using your templates and primers.

SNaPshot Primer Design See "SNaPshot Primer Design and Evaluation Recommendations" on page 29 for recommendations on designing and evaluating primers.

Pooling PCR Amplified SNaPshot Templates If you have multiple purified PCR amplified samples to run in a single SNaPshot reaction, mix equal volumes (*e.g.*, 2 µL each) of these products in a tube and place the tube on ice.

Note SNaPshot Multiplex Ready Mix gives satisfactory results over a range of 0.01 to 0.40 pmol of PCR products (depending on template) in a 10-µL reaction.

Note For a description of how to convert nanograms per microliter to picomoles per microliter, refer to Appendix B on page 31.

Pooling SNaPshot Primers All the primers to be used in a single SNaPshot reaction should be premixed to give a final concentration of 0.2 µM for each primer. Place the primer mixture on ice.

Note SNaPshot Multiplex Ready Mix has been designed to exhaust all primers in the reaction. The recommended starting concentration for each primer is 0.2 µM. If a particular primer has a consistently low or high signal, increase or decrease the concentration of that primer. Successful results have been obtained using primers with concentrations that range between 0.05 µM and 1 µM in a six-primer mixture. Adjusting the template concentration is usually not required.

Setting Up Your Sample Reaction

To set up your sample reaction:

Step	Action
1	Thaw the SNaPshot Multiplex Ready Reaction Mix **on ice**.

Note Adjust the volume of deionized water to accommodate any changes in primer or template volumes.

Note Make a master mix if you are running several samples containing common components.

Combine the following:

Item	Volume (µL/ Sample)
SNaPshot Multiplex Ready Reaction Mix	5
Pooled PCR products	3
Pooled SNaPshot primers	1
Deionized water	1
Total	10

2 Mix thoroughly and spin briefly.

Aliquot 10 µL into each MicroAmp tube/well.

Note It is important to keep the reaction mixture on ice before putting it into the thermal cycler. **Leaving the mixture at ambient temperature for 20 minutes or longer may lead to higher background.**

3 Proceed to "Thermal Cycling and Post-Extension Treatment" on page 16.

Thermal Cycling and Post-Extension Treatment

Overview This section describes how to conduct thermal cycling and how to remove unincorporated ddNTPs after thermal cycling.

Thermal Cycling To conduct thermal cycling:

Step	Action
1	Place the tubes in a GeneAmp 9600 thermal cycler, and set the volume to 10 µL.
2	Repeat the following for 25 cycles: ♦ Rapid thermal ramp to 96 °C ♦ 96 °C for 10 seconds ♦ Rapid thermal ramp to 50 °C ♦ 50 °C for 5 seconds ♦ Rapid thermal ramp to 60 °C ♦ 60 °C for 30 seconds **Note** Thermal cycling takes approximately 1 hour and 10 minutes to complete.
3	Rapid thermal ramp to 4 °C, and hold until ready for post-extension treatment.

Post-Extension Treatment **IMPORTANT** Left untreated, the unincorporated [F]ddNTPs will co-migrate with the fragment(s) of interest. Removal of the 5′ phosphoryl groups by phosphatase treatment alters the migration of the unincorporated [F]ddNTPs and thus prohibits interference.

To conduct post-extension treatment:

Step	Action
1	Add one of the following to the reaction mixture, mix thoroughly, and incubate at 37 °C for 1 hour. **Note** Because of the high glycerol concentration in the undiluted SAP, vortex briefly to mix. ♦ 1.0 Unit of Shrimp Alkaline Phosphatase (SAP) or ♦ 1.0 Unit of Calf Intestinal Phosphatase (CIP)

To conduct post-extension treatment: *(continued)*

Step	Action
2	Deactivate the enzyme by incubating at 75 °C for 15 minutes.
3	Samples may be placed at 4 °C for up to 24 hours prior to electrophoresis on the 310/3100/3700 systems. For storage longer than 24 hours, store the samples at –20 °C.
4	

If you are running an...	Then proceed to...
ABI PRISM 310 Genetic Analyzer	"Electrophoresis on the ABI PRISM 310 Genetic Analyzer" on page 18.
ABI PRISM 3100 Genetic Analyzer	"Electrophoresis on the ABI PRISM 3100 Genetic Analyzer" on page 22.
ABI PRISM 3700 DNA Analyzer	"Electrophoresis on the ABI PRISM 3700 DNA Analyzer" on page 24.

Electrophoresis on the ABI PRISM 310 Genetic Analyzer

Overview

This section describes electrophoresis of SNaPshot products on the ABI PRISM 310 Genetic Analyzer using the 310 Data Collection version 2.1.

Note For more information about using the ABI PRISM 310 Genetic Analyzer, refer to the *ABI PRISM 310 Genetic Analyzer User's Manual* (P/N 903565).

The Polymer

The SNaPshot kits may be used with:

- POP-4™ polymer, in conjunction with GS POP-4 (1mL) E5 module

⚠ CAUTION **CHEMICAL HAZARD. POP-4 polymer** may cause eye, skin, and respiratory tract irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Use for research and development purposes only.

GeneScan E5 Run Module Parameters

The GeneScan E5 Run Module encodes the following parameters on the 310 instrument:

Parameter	Control Module GS POP-4 (1mL) E5
Injection time	5 seconds
Electrophoresis voltage	15 kV
Collection time	24 minutes
EP voltage	15 kV
Heat plate temperature	60 °C
Syringe pump time	150 seconds
Preinjection EP	120 seconds

Adjusting the Run Time

Depending upon primer length, the peaks of interest may appear well before the run ends. For this reason, you may want to shorten the collection time.

Adjusting the Injection Time for Signal Variability

If increased or decreased signal is routinely observed, you may want to decrease or increase injection times, respectively. For a description of

how to adjust the injection time on the 310 Genetic Analyzer, refer to the *ABI PRISM 310 Genetic Analyzer User's Manual* (P/N 903565).

Running Matrix Standards

If you are running the ABI PRISM SNaPshot Multiplex Kit reactions for the first time, you will need a Matrix Standard Set DS-02 [dR110, dRGG, dTAMRA, dROX, LIZ] for the 310 Genetic Analyzer system. Run the ABI PRISM DS-02 Matrix Standards Kit (P/N 4323050), along with the other control and sample reactions.

Refer to the DS-02 Matrix Standards Kit product insert for directions on how to prepare the DS-02 matrix standards.

Preparing Samples

Follow the instructions below if you are using an ABI PRISM 310 Genetic Analyzer to run your samples.

To prepare samples for the 310 Genetic Analyzer:

Step	Action
1	Thaw Hi-Di formamide, SNaPshot products, and the GeneScan-120 LIZ size standard. Vortex to mix and spin briefly. **⚠ WARNING** **CHEMICAL HAZARD. Formamide** is harmful if absorbed through the skin and may cause irritation to the eyes, skin, and respiratory tract. It may cause damage to the central nervous system and the male and female reproductive systems, and is a possible birth defect hazard. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
2	Add 9 µL of Hi-Di formamide into each tube.
3	Add 0.5 µL of the SNaPshot product and 0.5 µL GeneScan-120 LIZ size standard into each tube. **Note** Total volume for injection is 10 µL **Note** Dilute the control reaction 1:2. Mix 0.5 µL of the diluted products with 0.5 µL of GeneScan-120 LIZ size standard. **Note** If you want to use volumes greater than 0.5 µL, the following mixing steps are suggested: a. Dilute 2 µL of SNaPshot product in 6 µL of Hi-Di formamide b. Dilute 2 µL of GeneScan-120 LIZ in 6 µL of Hi-Di formamide (enough for 4 samples) c. Mix: – 2 µL of diluted SNaPshot product – 2 µL of diluted GeneScan-120 LIZ size standard – 6 µL of Hi-Di formamide
4	Vortex briefly and quick spin.
5	Denature the samples by placing them at 95 °C for 5 minutes.
6	Place the samples on ice or at 4 °C until you are ready to load them on the 310 Genetic Analyzer.
7	Quick spin or tap the tubes or plates to bring liquid to the bottom of the tubes.

To prepare samples for the 310 Genetic Analyzer: *(continued)*

Step	Action
8	Refer to the *ABI PRISM 310 User's Manual* for specific directions on the following: a. Verify that you have chosen GeneScan Run Module E5. b. Confirm the injection time. c. Verify that you have selected the DS-02 GeneScan Matrix Set for the 310 Genetic Analyzer system. d. Verify that you have selected the GeneScan-120 LIZ size standard analysis parameter for automatic data analysis. **Note** To set up the GeneScan-120 LIZ size standard automatic analysis, refer to the instructions in the GeneScan-120 LIZ size standard product insert.

Electrophoresis on the ABI PRISM 3100 Genetic Analyzer

Setting Up the Analyzer

Before any run, make sure that the 3100 Genetic Analyzer is set up with a 36-cm capillary array and POP-4 polymer.

Running Matrix Standards

If you are running the ABI PRISM SNaPshot Multiplex Kit reactions for the first time, you will need a Matrix Standard Set DS-02 [dR110. dRGG, dTAMRA, dROX, LIZ] for the 3100 Genetic Analyzer (P/N 4323014).

Refer to the DS-02 Matrix Standards Kit product insert for directions on how to prepare the DS-02 matrix standards.

Preparing the Samples

To prepare samples for the 3100 Genetic Analyzer:

Step	Action
1	Thaw Hi-Di formamide, SNaPshot products, and the GeneScan-120 LIZ size standard. Vortex to mix and spin briefly.

⚠ WARNING CHEMICAL HAZARD. Formamide is harmful if absorbed through the skin and may cause irritation to the eyes, skin, and respiratory tract. It may cause damage to the central nervous system and the male and female reproductive systems, and is a possible birth defect hazard. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

2 Add 9 µL of Hi-Di formamide to each well.

3 Add 0.5 µL of the SNaPshot products and 0.5 µL of GeneScan-120 LIZ size standard into each well and seal the plates.

Note Total volume for injection is 10 µL.

Note If you want to use volumes greater than 0.5 µL, the following mixing steps are suggested:

a. Dilute 2 µL of SNaPshot product in 6 µL of Hi-Di formamide.

b. Dilute 2 µL of GS120 in 6 µL of Hi-Di formamide (enough for four samples).

c. Mix.

- 2 µL of diluted SNaPshot product
- 2 µL of diluted GeneScan-120 LIZ size standard
- 6 µL of Hi-Di formamide

To prepare samples for the 3100 Genetic Analyzer: *(continued)*

Step	Action
4	Vortex briefly and spin briefly.
5	Denature the samples by placing them at 95 °C for 5 minutes.
6	Place the samples on ice or at 4 °C until you are ready to load the analyzer.

GeneScan Run Parameters

To start the run:

Step	Action
1	In the **New Plate** setup, select **Dye Set E5** and SNP36_POP4 default module.
2	Start the run.

Note To set up the GeneScan-120 LIZ size standard automatic analysis, refer to the instructions in the GeneScan-120 LIZ size standard product insert.

Electrophoresis on the ABI PRISM 3700 DNA Analyzer

Setting Up the Analyzer

Before any run, make sure that the 3700 DNA Analyzer is set up with a 50-cm capillary array and POP-5 polymer.

Running Matrix Standards

If you are running the ABI PRISM SNaPshot Multiplex Kit reactions for the first time, you will need a Matrix Standard Set DS-02 for the 3700 DNA Analyzer (P/N 4323785).

Refer to the DS-02 Matrix Standards Kit product insert for directions on how to prepare the DS-02 matrix standards.

Preparing the Samples

To prepare samples for the 3700 DNA Analyzer:

Step	Action
1	Thaw Hi-Di formamide, SNaPshot products, and the GeneScan-120 LIZ size standard. Vortex to mix and spin briefly.
	⚠ WARNING CHEMICAL HAZARD. Formamide is harmful if absorbed through the skin and may cause irritation to the eyes, skin, and respiratory tract. It may cause damage to the central nervous system and the male and female reproductive systems, and is a possible birth defect hazard. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
2	Add 9 µL of Hi-Di formamide to each well.
3	Add 0.5 µL of SNaPshot products and 0.5 µL of GeneScan-120 LIZ size standard to each well, and seal the plates.
	Note Total volume for injection is 10 µL.
	Note If you want to use volumes greater than 0.5 µL, the following mixing steps are suggested:
	a. Dilute 2 µL of SNaPshot product in 6 µL of Hi-Di formamide.
	b. Dilute 2 µL of GeneScan-120 LIZ in 6 µL of Hi-Di formamide (enough for four samples).
	c. Mix:
	– 2 µL of diluted SNaPshot product
	– 2 µL of diluted GeneScan-120 LIZ size standard
	– 6 µL of Hi-Di formamide
4	Vortex briefly and spin briefly.

To prepare samples for the 3700 DNA Analyzer: *(continued)*

Step	Action
5	Denature the samples by placing them at 95 °C for 5 minutes. Place the samples on ice or at 4 °C until you are ready to load the analyzer.

Setting Up GeneScan Parameters

Setting up the GeneScan application:

Step	Action
1	In the **New Plate** setup, select **Dye Set E5** and **SNP1_1POP5 module** **Note** Data collection time in the default SNP1_1POP5 module is 900 seconds. To ensure that all 9 peaks in GeneScan-120 LIZ size standard are collected, extend the data collection time to 1100 seconds. **Note** If the signal variation from the left to the right side of the array becomes a concern, try lowering the running voltage to 6 KV. You will also need to extend the data delay time from 900 seconds to 1200 seconds and the data collection time from 900 seconds to 1800 seconds (refer to step 3 for information on modifying the module). **Note** To set up the GeneScan-120 LIZ size standard automatic analysis, refer to the instructions in the GeneScan-120 LIZ size standard product insert.
2	Start the run.

Setting up the GeneScan application: *(continued)*

Step	Action
3	Use the table below to adjust the signal intensity using the **Module Editor**.

Observation	Possible Cause	Recommended Action
Signal varies across the capillary array	The run temperature and the run voltage need adjusting	Adjust the run conditions (in the following order): a. Lower the temperature to 50 °C b. Lower the run voltage to 6 KV c. Increase the data delay and run times to accommodate the slower run times caused by a. and b. above
	The cuvette temperature is not optimized	Test in increments of 5 °C through the range 35–50 °C until you identify the temperature that produces the best signal uniformity across the array

Data Analysis

Overview This section describes how to perform GeneScan data analysis.

Analyzing Sample Files on the 310 Instrument Analyze the files using GeneScan Analysis Software version 3.1 and GeneScan-120 LIZ size standard analysis parameter files. For a detailed explanation, refer to the *ABI PRISM GeneScan Analysis Software User's Manual* (P/N 4303242) and the GeneScan-120 LIZ size standard product insert.

Analyzing Sample Files on the 3100 and 3700 Instruments Analyze the files using GeneScan Analysis Software version 3.5 and GeneScan-120 LIZ size standard analysis parameter files. For a detailed explanation, refer to the *ABI PRISM GeneScan Analysis Software User's Manual* (P/N 4303242) and the GeneScan-120 LIZ size standard product insert.

Example of Control Reaction

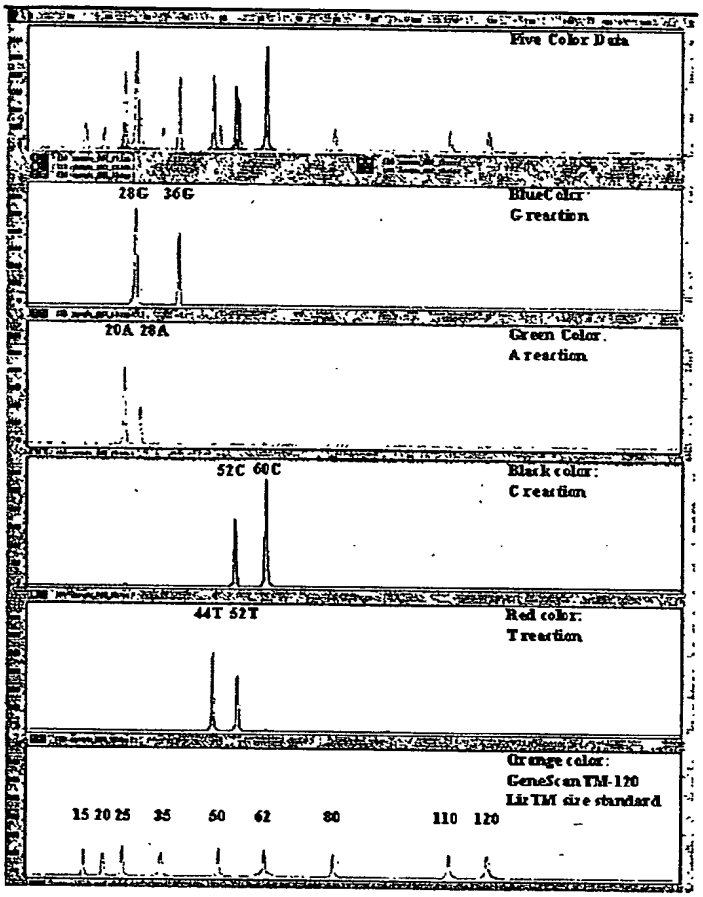


Figure 1 Electropherogram of the Multiplex Control Reaction products along with GeneScan™-120 LIZ™ size standard

Allele Calling Genotyper 3.7 can be used to analyze this data. Please refer to the *Genotyper User's Manual* for more information. Applied Biosystems is constantly developing new software solutions particularly for SNP analysis. Please check our web site.

Appendix A. SNaPshot Primer Design and Evaluation Recommendations

Follow these recommendations for designing and evaluating primers:

- Primers included in a single reaction need to differ significantly in lengths in order to avoid overlap between the final SNaPshot products. A difference of 4–6 nucleotides between primer lengths is recommended as a starting point.
- The length of a primer can be modified by the addition of nonhomologous polynucleotides at the 5′ end. Since the recommended annealing temperature for a SNaPshot control primer is 50 °C, the melting temperature for the complementary region between any primer and its corresponding template should be at least 50 °C.
- Poly (dT), poly (dA), poly (dC), and poly (dGACT) are 5′ non-homologous tails which are predicted to have minimal secondary structures. They have all been used successfully. Generally the signal patterns are not affected by the kinds of tails that are used. The 5′ poly (dT) tails however may interfere with the addition of 3′ ddA.
- The mobility of an oligonucleotide in capillary electrophoresis is determined by its size, nucleotide composition, and dye. Thus the effect of nucleotide composition on mobility can be significant when the primer is short. We strongly recommend that primers shorter than 36 nucleotides be tested before being multiplexed to ensure that the final products are spatially resolved when analyzed on the instrument.
- Check primers for possible extendable hairpin structures within each primer and for extendable dimer formation between primers.
- HPLC purification of primers is recommended for oligonucleotides longer than 30 nucleotides. Heterogenous primer mixtures containing mixed molecular weight oligonucleotides may yield undesired products that will confuse analysis.
- Since SNP interrogation using primer extension does not permit any flexibility with respect to the location of the 3′ end of the primer, use primers that are complementary to the negative (–) DNA strand if the positive (+) DNA strand is difficult to assay.

- Run a negative control reaction (lacking template DNA) when evaluating a new primer.
- Certain primer/template combinations may require adjusting the annealing temperature or annealing time. Refer to Appendix C on page 32.
- For an illustration of the use of multiplexed primers in a SNP validation application see the following reference:

Lindblad-Toh, K., *et al.* Large -scale discovery and genotyping of single-nucleotide polymorphisms in the mouse. 2000, *Nature Genetics* 24: 381–386.

Appendix B. Converting Nanograms to Picomoles

Procedure To convert nanograms per microliter (A_{260}) to picomoles per microliter:

Step	Action
1	Measure the absorbance of your sample and multiply by a dilution factor.
	a. Using a spectrophotometer, measure the DNA sample absorbance at 260 nm (A_{260}). It may be necessary to dilute the sample for an accurate measurement.
	b. Multiply the A_{260} by any dilution factor used.
	For example, if the A_{260} sample reading is 0.060 and the dilution factor is 10, then:
	$A_{260} = 0.060 \times 10 = 0.600$
2	Multiply the A_{260} value by 50 µg/mL (50 ng/µL) to obtain nanograms per microliter of double-stranded DNA.
	For example, if the A_{260}=0.600, then:
	0.600 x 50 µg/mL= 30 ng/µL
	Note 1.0 OD =50 µg/mL of double-stranded DNA
3	Determine the molecular weight of the PCR product by multiplying the number of base pairs by 650 daltons/base pair.
	For example, if the oligo is 120 base pairs in length, then:
	120 x 650 Da/bp = 78,000 Da
4	Convert nanograms per microliter to picomoles per microliter by
	a. dividing the molecular weight into 10^3
	b. multiplying by the concentration determined in step 2
	For example:
	(10^3 / 78,000 Da) x 30 ng/µL = 0.38 pmol/µL

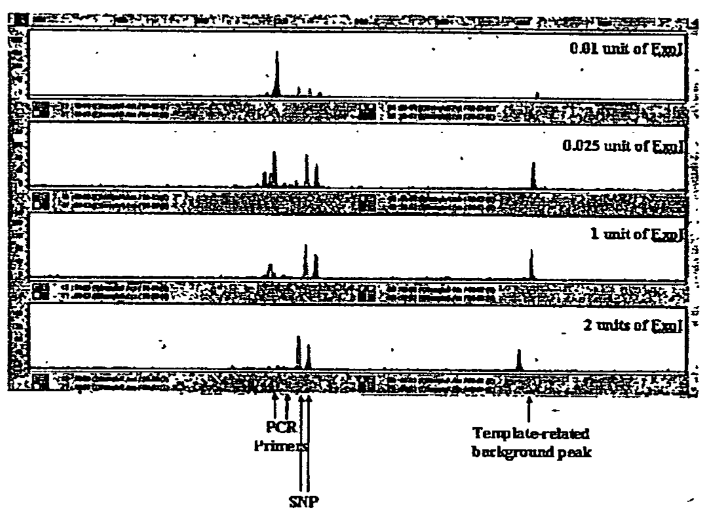
Appendix C. Troubleshooting

Troubleshooting Low Signal Here are some possible causes of Low Signal

Observation	Possible Cause(s)	Recommended Action
Low signal	Insufficient concentration of annealed primer, possibly because of low annealing and extension efficiency.	Increase the primer concentration to 1 pmol per reaction. Combined primer concentrations greater then 4 pmol are not recommended as they may cause ddNTP mis-incorporation.
	Suboptimized thermal cycling conditions Primers annealing to templates occur at a much slower rate than that of ddNTP incorporation by Taq DNA Polymerase at the suggested temperature.	If you consistently observe low signals, try optimizing the annealing temperature and/or the annealing time. The annealing temperature may be the same as the extension temperature.
	Insufficient amplification of template DNA	Measure the absorbance of the DNA template at 260 nm to confirm the DNA concentration in the amplification products. Satisfactory results have been obtained using 0.01 pmol of DNA template per reaction. **Note** This is a less likely cause of low signal than insufficient concentration of primers.
	Inappropriate injection time.	Increase the injection time.

Troubleshooting Extraneous Peaks Use the following table to troubleshoot extraneous peaks:

Causes of Extraneous Peaks

Observation	Possible Cause(s)	Recommended Action
Extraneous peaks	Incomplete removal of PCR primers. **Note** PCR primers that have not been removed can participate in the SNaPshot primer extension reaction and resemble signal derived from the SNaPshot interrogation primer.	Since the primer size and sequence is known, look at the data to determine if the peak observed is the expected size (primer + 1 nt) and color of the expected peak.
		Use fresh SAP and *Exo* I, or employ an alternative method of primer removal. **Note** There have been some reports of PCR primers that are refractive to digestion by *Exo* I. See Figure 1.
	 **Figure 2** Electropherogram of SNaPshot products.Increasing the amount of *Exo* I resulted in less peaks from PCR primers.	
	Incomplete removal of [F]ddNTPs by SAP digestion results in comigration of [F]ddNTPs with the fragments of interest.	The undigested [F]ddNTPs normally appear as peaks larger than 70 bp. Excess [F]ddNTPs also result in peaks of smaller sizes. In this case, use fresh CIP or SAP.

Causes of extraneous peaks (*continued*):

Observation	Possible Cause	Recommended Action
Extraneous peaks	PCR-amplified templates. **Note** These products are usually longer than 60 base pairs.	To determine if the peaks are from templates, run a SNaPshot reaction using the templates without SNaPshot primers. Any peaks that appear will be from the PCR amplification of the templates. To decrease the amount of these extraneous peaks, try decreasing the amount of *Exo* I used. If you are using column purification, try a more stringent elution condition to minimize short fragment recovery. Alternatively you can decrease the concentration of templates in the SNaPshot reaction. See Figure 3.

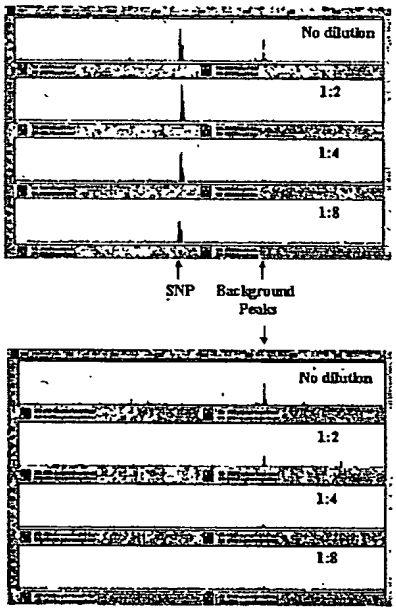


Figure 3 Electropherogram of SNaPshot products. Two microliters of PCR-amplified templates (undiluted, 1:2 diluted, 1:4 diluted, 1:8 diluted) was used in the SNaPshot reaction with a SNaPshot primer (top panel) or without a SNaPshot primer (bottom panel). Background peaks (green peaks) are the same in samples with or without SNaPshot primers and decrease as the templates become more diluted.

Causes of extraneous peaks (*continued*);

Observation	Possible Cause	Recommended Action
Extraneous peaks that resemble a conventional Sanger sequencing reaction. The peak of interest has significantly reduced amplitude.	Incomplete removal of dNTPs from PCR reactions. This enables dNTPs to participate in the ddNTP extension reaction. Refer to Figure 4.	Use fresh SAP. Use an alternate means of PCR reaction purification such as those listed on page 9 under PCR Purification Kits.

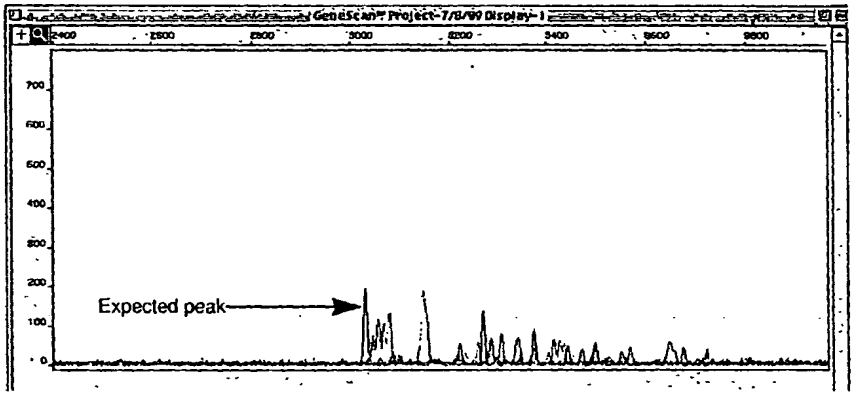


Figure 4 Electropherogram resulting from the presence of residual dNTPs.

Extraneous peaks	Primer hairpin extension.	Carefully analyze the primer sequence.
	Primer dimer extension.	♦ Avoid using primers that are capable of annealing to themselves and leaving a recessed 3′ end. ♦ Use primer analysis software to help identify problems associated with primer design. ♦ Try designing primers using the complementary DNA strand.

Troubleshooting Sizing Problems Use the following table to troubleshoot sizing problems.

Observation	Possible Cause	Recommended Action
The fragment sizes observed are different from the expected sizes	Incorporation of dye greatly effects the mobility of the extension products. Often shorter fragments will appear to be nearly five bases longer than their actual size.	No action required.
The sizes of identical fragments vary between runs or capillaries.	Size standard improperly called due to low-intensity peaks being called instead of the real peaks.	Reanalyze the samples after increasing the minimum peak height value. Make sure that you change the analysis parameter settings used for your analysis (refer to the *GeneScan User's Manual* for more information).
	Off-scale peaks in the region of a size standard peak is causing that size standard peak to fail to be recognized. Older versions of GeneScan (earlier than GS 3.52) have a size matching algorithm that can cause this problem.	Get the new patch from the Applied Biosystems web site.

Appendix D. Technical Support

Contacting Technical Support

You can contact Applied Biosystems for technical support by telephone or fax, by e-mail, or through the Internet. You can order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents 24 hours a day. In addition, you can download documents in PDF format from the Applied Biosystems Web site (please see the section "To Obtain Documents on Demand" following the telephone information below).

To Contact Technical Support by E-Mail

Contact technical support by e-mail for help in the following product areas:

Product Area	E-mail address
Genetic Analysis (DNA Sequencing)	galab@appliedbiosystems.com
Sequence Detection Systems and PCR	pcrlab@appliedbiosystems.com
Protein Sequencing, Peptide and DNA Synthesis	corelab@appliedbiosystems.com
Biochromatography, PerSeptive DNA, PNA and Peptide Synthesis systems, CytoFluor®, FMAT™, Voyager™, and Mariner™ Mass Spectrometers	tsupport@appliedbiosystems.com
Applied Biosystems/MDS Sciex	api3-support@sciex.com
Chemiluminescence (Tropix)	tropix@appliedbiosystems.com

Hours for Telephone Technical Support

In the United States and Canada, technical support is available at the following times:

Product	Hours
Chemiluminescence	8:30 a.m. to 5:30 p.m. Eastern Time
Framingham support	8:00 a.m. to 6:00 p.m. Eastern Time
All Other Products	5:30 a.m. to 5:00 p.m. Pacific Time

To Contact Technical Support by Telephone or Fax

In North America

To contact Applied Biosystems Technical Support, use the telephone or fax numbers given below. (To open a service call for other support needs, or in case of an emergency, dial **1-800-831-6844** and press **1**.)

Product or Product Area	Telephone Dial...	Fax Dial...
ABI PRISM® 3700 DNA Analyzer	**1-800-831-6844**, then press **8**	**1-650-638-5981**
DNA Synthesis	**1-800-831-6844**, then press **21**	**1-650-638-5981**
Fluorescent DNA Sequencing	**1-800-831-6844**, then press **22**	**1-650-638-5981**
Fluorescent Fragment Analysis (includes GeneScan® applications)	**1-800-831-6844**, then press **23**	**1-650-638-5981**
Integrated Thermal Cyclers (ABI PRISM®877 and Catalyst 800 instruments)	**1-800-831-6844**, then press **24**	**1-650-638-5981**
ABI PRISM® 3100 Genetic Analyzer	**1-800-831-6844**, then press **26**	**1-650-638-5981**
BioInformatics (includes BioLIMS®, BioMerge®, and SQL GT™ applications)	**1-800-831-6844**, then press **25**	**1-505-982-7690**
Peptide Synthesis (433 and 43X Systems)	**1-800-831-6844**, then press **31**	**1-650-638-5981**
Protein Sequencing (Procise® Protein Sequencing Systems)	**1-800-831-6844**, then press **32**	**1-650-638-5981**
PCR and Sequence Detection	**1-800-762-4001**, then press **1** for PCR, **2** for the 7700 or 5700, **6** for the 6700 or dial **1-800-831-6844**, then press **5**	**1-240-453-4613**

Product or Product Area	Telephone Dial...	Fax Dial...
Voyager™ MALDI-TOF Biospectrometry and Mariner™ ESI-TOF Mass Spectrometry Workstations	1-800-899-5858, then press 13	1-508-383-7855
Biochromatography (BioCAD® Workstations and Poros® Perfusion Chromatography Products)	1-800-899-5858, then press 14	1-508-383-7855
Expedite™ Nucleic acid Synthesis Systems	1-800-899-5858, then press 15	1-508-383-7855
Peptide Synthesis (Pioneer™ and 9050 Plus Peptide Synthesizers)	1-800-899-5858, then press 15	1-508-383-7855
PNA Custom and Synthesis	1-800-899-5858, then press 15	1-508-383-7855
FMAT™ 8100 HTS System and CytoFluor® 4000 Fluorescence Plate Reader	1-800-899-5858, then press 16	1-508-383-7855
Chemiluminescence (Tropix)	1-800-542-2369 (U.S. only), or 1-781-271-0045	1-781-275-8581
Applied Biosystems/MDS Sciex	1-800-952-4716	1-650-638-6223

Outside North America

Region	Telephone Dial...	Fax Dial...
Africa and the Middle East		
Africa (English Speaking) and West Asia (Fairlands, South Africa)	27 11 478 0411	27 11 478 0349
South Africa (Johannesburg)	27 11 478 0411	27 11 478 0349
Middle Eastern Countries and North Africa (Monza, Italia)	39 (0)39 8389 481	39 (0)39 8389 493

Region	Telephone Dial...	Fax Dial...
Eastern Asia, China, Oceania		
Australia (Scoresby, Victoria)	61 3 9730 8600	61 3 9730 8799
China (Beijing)	86 10 64106608	86 10 64106617
Hong Kong	852 2756 6928	852 2756 6968
Korea (Seoul)	82 2 593 6470/6471	82 2 593 6472
Malaysia (Petaling Jaya)	60 3 758 8268	60 3 754 9043
Singapore	65 896 2168	65 896 2147
Taiwan (Taipei Hsien)	886 2 2358 2838	886 2 2358 2839
Thailand (Bangkok)	66 2 719 6405	66 2 319 9788
Europe		
Austria (Wien)	43 (0)1 867 35 75 0	43 (0)1 867 35 75 11
Belgium	32 (0)2 712 5555	32 (0)2 712 5516
Czech Republic and Slovakia (Praha)	420 2 61 222 164	420 2 61 222 168
Denmark (Naerum)	45 45 58 60 00	45 45 58 60 01
Finland (Espoo)	358 (0)9 251 24 250	358 (0)9 251 24 243
France (Paris)	33 (0)1 69 59 85 85	33 (0)1 69 59 85 00
Germany (Weiterstadt)	49 (0) 6150 101 0	49 (0) 6150 101 101
Hungary (Budapest)	36 (0)1 270 8398	36 (0)1 270 8288
Italy (Milano)	39 (0)39 83891	39 (0)39 838 9492
Norway (Oslo)	47 23 12 06 05	47 23 12 05 75
Poland, Lithuania, Latvia, and Estonia (Warszawa)	48 (22) 866 40 10	48 (22) 866 40 20
Portugal (Lisboa)	351 (0)22 605 33 14	351 (0)22 605 33 15
Russia (Moskva)	7 095 935 8888	7 095 564 8787
South East Europe (Zagreb, Croatia)	385 1 34 91 927	385 1 34 91 840
Spain (Tres Cantos)	34 (0)91 806 1210	34 (0)91 806 1206
Sweden (Stockholm)	46 (0)8 619 4400	46 (0)8 619 4401
Switzerland (Rotkreuz)	41 (0)41 799 7777	41 (0)41 790 0676
The Netherlands (Nieuwerkerk a/d IJssel)	31 (0)180 331400	31 (0)180 331409

Region	Telephone Dial...	Fax Dial...
United Kingdom (Warrington, Cheshire)	44 (0)1925 825650	44 (0)1925 282502
All other countries not listed (Warrington, UK)	44 (0)1925 282481	44 (0)1925 282509
Japan		
Japan (Hacchobori, Chuo-Ku, Tokyo)	81 3 5566 6006	81 3 5566 6505
Latin America		
Del.A. Obregon, Mexico	305-670-4350	305-670-4349

To Reach Technical Support Through the Internet

We strongly encourage you to visit our Web site for answers to frequently asked questions and for more information about our products. You can also order technical documents or an index of available documents and have them faxed or e-mailed to you through our site. The Applied Biosystems Web site address is

http://www.appliedbiosystems.com/techsupp

To submit technical questions from North America or Europe:

Step	Action
1	Access the Applied Biosystems Technical Support Web site.
2	Under the **Troubleshooting** heading, click **Support Request Forms**, then select the relevant support region for the product area of interest.
3	Enter the requested information and your question in the displayed form, then click **Ask Us RIGHT NOW** (blue button with yellow text).
4	Enter the required information in the next form (if you have not already done so), then click **Ask Us RIGHT NOW.** You will receive an e-mail reply to your question from one of our technical experts within 24 to 48 hours.

To Obtain Documents on Demand

Free, 24-hour access to Applied Biosystems technical documents, including MSDSs, is available by fax or e-mail or by download from our Web site.

To order documents...	Then...
by index number	a. Access the Applied Biosystems Technical Support Web site at **http://www.appliedbiosystems.com/techsupp** b. Click the **Index** link for the document type you want, then find the document you want and record the index number. c. Use the index number when requesting documents following the procedures below.
by phone for fax delivery	a. From the U.S. or Canada, call **1-800-487-6809**, or from outside the U.S. and Canada, call **1-858-712-0317**. b. Follow the voice instructions to order the documents you want. **Note** There is a limit of five documents per request.
through the Internet for fax or e-mail delivery	a. Access the Applied Biosystems Technical Support Web site at **http://www.appliedbiosystems.com/techsupp** b. Under **Resource Libraries**, click the type of document you want. c. Enter or select the requested information in the displayed form, then click **Search**. d. In the displayed search results, select a check box for the method of delivery for each document that matches your criteria, then click **Deliver Selected Documents Now** (or click the PDF icon for the document to download it immediately). e. Fill in the information form (if you have not previously done so), then click **Deliver Selected Documents Now** to submit your order. **Note** There is a limit of five documents per request for fax deliver but no limit on the number of documents you can order for e-mail delivery.

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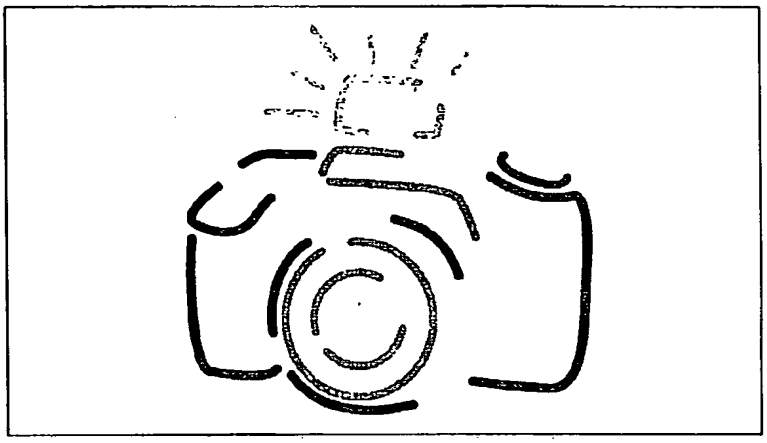
Foster City, California 94404-1128
USA

P/N 4323357 Rev. A

Applied Biosystems

ABI PRISM® SNaPshot™ Multiplex System

- **SNP validation, linkage mapping, and association studies**
- **Up to 10 primer/template combinations multiplexed during single-base extension**
- **SNP interrogation and automatic genotype assignment**



The ABI PRISM® SNaPshot™ Multiplex System includes:
- **ABI PRISM SNaPshot Multiplex Kit**
- **GeneScan™-120 LIZ™ Size Standard**
- **Genotyper® Software v 3.7**
- **Matrix Standard Set DS-02**

Introduction

Single nucleotide polymorphisms (SNPs) are used in the study of complex genetic diseases, Mendelian traits, population studies, and pharmacogenetics. Academic and pharmaceutical researchers must map genetic variation in their search to identify disease genes. Recent sequencing projects have revealed millions of potential SNPs that could be characterized and used to construct detailed genetic maps

The SNP analysis sequence involves three phases. SNP discovery, validation and scoring. During the discovery phase, researchers identify potential SNPs by either de novo sequencing or thorough comparison of sequences residing in databases (in silico discovery) SNP validation confirms the SNPs as "real" (non-sequencing errors). SNP subsets are then validated for an allele frequency that is useful in mapping (usually between 20% and 40% in the population in question). The SNP scoring phase includes linkage analysis and association studies.

A Complete SNP Solution for SNP Validation, Linkage Mapping, and Association Studies

The ABI PRISM® SNaPshot™ Multiplex System enables ABI PRISM capillary electrophoresis instruments to perform SNP analyses from high-throughput SNP validation through to medium-throughput SNP scoring (linkage analysis and association studies). The SNaPshot Multiplex Kit multiplexes up to 10 single-base, primer-extension reactions in one reaction (Table 1). The GeneScan™-120 LIZ™ Size Standard eliminates the need for manual allele calls of the multiplexed samples when used with Genotyper® Software v 3 7. The result is a complete system that contains everything required for precise and cost-effective SNP analysis.

Accurate, Automated Five-Dye SNP Analysis

The ABI PRISM SNaPshot Multiplex System provides investigators with the necessary reagents and software to perform SNP analysis on their existing capillary electrophoresis platforms. The kit interrogates up to 10 SNPs per reaction. Researchers can analyze more than 23,000 SNP genotypes per day on just one ABI PRISM® 3700 DNA Analyzer (Table 1). SNPs can be interrogated regardless of their position on the chromosome or separation from a neighboring SNP locus. User-defined, unlabeled primers enable the investigator to incorporate SNPs of interest cost-effectively, while the Multiplex Ready Reaction Mix provides robust analyses of multiplexed samples

Applied Biosystems

ABI Prism Instrument	SNP Throughput*			Application		
	Per Day	5-day wk	7-day wk	SNP validation	linkage mapping	association studies
310 Genetic Analyzer	480	2,400	3,360	X	X	X
3100 Genetic Analyzer	7,680	38,400	53,760	X	X	X
3700 DNA Analyzer	23,040	115,200	161,280	X	X	X

*Estimates based on daily, 24-hour operation, 10 SNPs/capillary

Table 1. SNaPshot Multiplex System—throughput and applications.

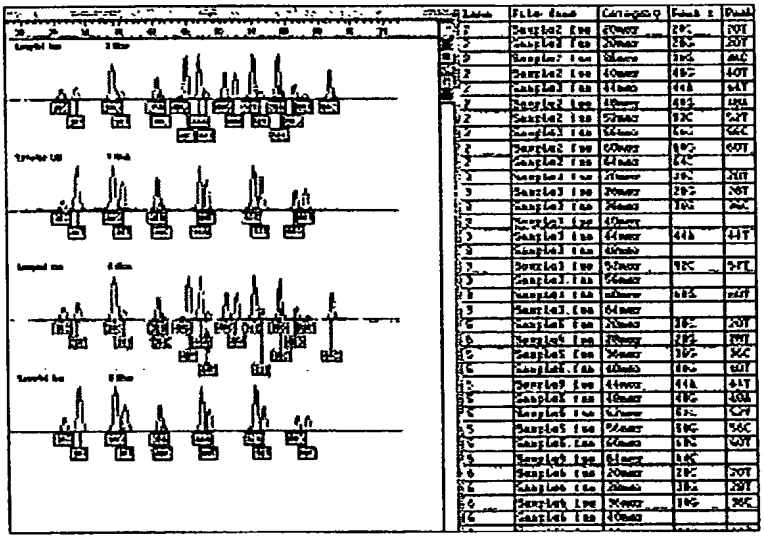


Figure 1. Automatic genotype assignment eliminates manual allele calls.

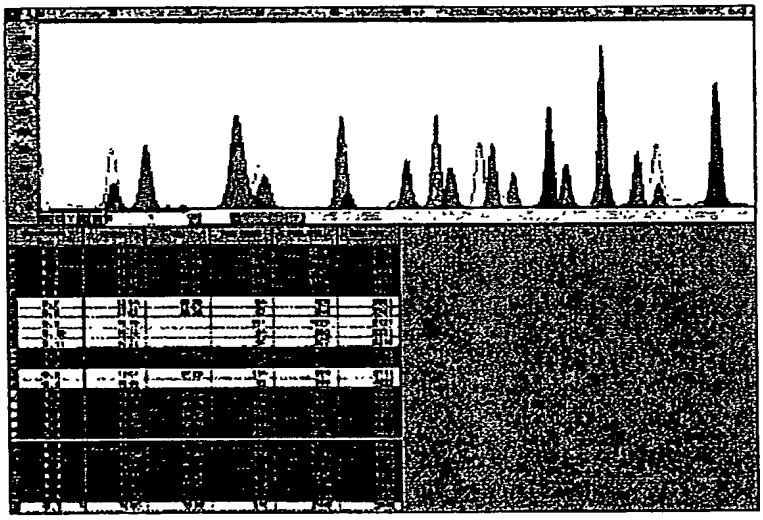


Figure 2. Multiplex 5-dye interrogation on the ABI Prism 3100 Genetic Analyzer.

Rapid Automatic Genotype Assignment

Sample sizing is easy with the GeneScan-120 LIZ Size Standard. It accurately sizes samples ranging from 20 to 110 nucleotides. When used with Genotyper Software v 3.7, the GeneScan-120 LIZ Size Standard eliminates the need for manual allele calls.

Genotyper Software v 3.7 provides rapid automatic genotype assignment and management of data generated from the SNaPshot Multiplex Kit. The Matrix Standard Set DS-02 (dR110, dR6G, dTAMRA™, dROX™, and LIZ™ dyes) ensures successful five-dye SNP analysis on all ABI Prism® genetic analyzers (Table 2).

High SNP Throughput on ABI Prism Genetic Analyzers

With its capability to multiplex up to 10 primer/template combinations simultaneously, the SNaPshot Multiplex System provides high throughput for SNP validation, as well as enough power for medium-throughput linkage analysis and association studies. Table 1 shows the SNP throughput and applications for the ABI Prism® 3700, 3100, and 310 analyzers.

dRhodamine Terminator Set and Size Standard		
ddNTP	dye	color
A	dR6G	green
C	dTAMRA™	yellow (black)
G	dR110	blue
U (T)	dROX™	red
size standard	LIZ™	orange

Table 2. SNaPshot Multiplex Kit 5-Dye Set

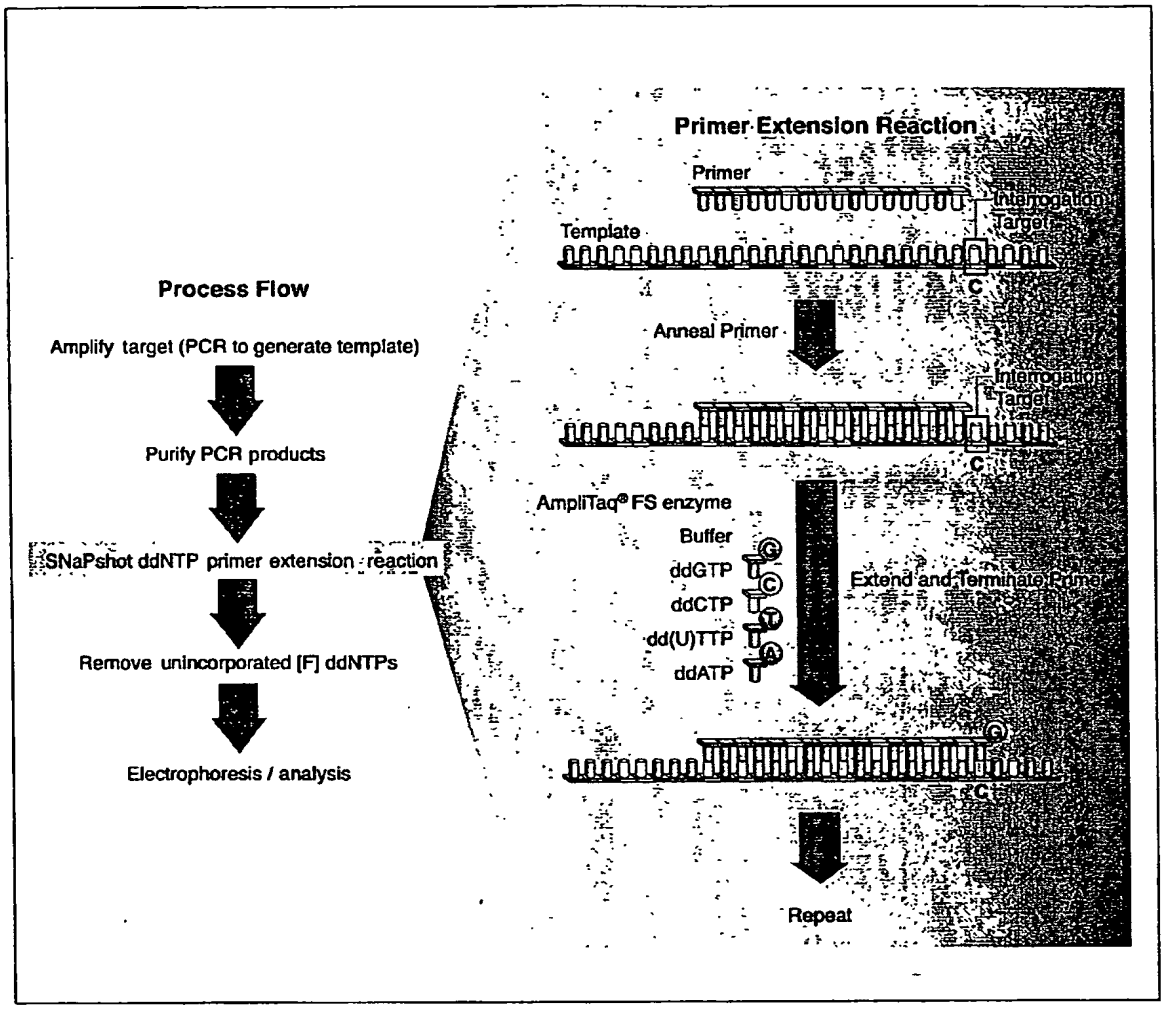


Figure 3. SNaPshot Multiplex Kit process flow and primer extension reaction

Ordering Information

SNaPshot™ Multiplex Kits	10-Plex Quantity	P/N
100-reaction kit with protocol	1,000 genotypes	4323151
1,000-reaction kit with protocol	10,000 genotypes	4323154
5,000 reaction kit with protocol	50,000 genotypes	4323155
SNaPshot Multiplex Kits*		
100-reaction kit*	1,000 genotypes	4323159
1,000-reaction kit*	10,000 genotypes	4323161
5,000-reaction kit*	50,000 genotypes	4323163

Size Standard (sold separately)	Comments	P/N
GeneScan™-120 LIZ™ Size Standard**	800 capillary runs	4324287
Reagents and Software (sold separately)		
Matrix Standard Set DS-02	For ABI PRISM 3700 DNA Analyzer	4323785
Matrix Standard Set DS-02	For ABI PRISM 3100 Genetic Analyzer	4323014
Matrix Standard Set DS-02	For ABI PRISM 310 Genetic Analyzer	4323050
Genotyper Software v 3.7	Initial license	4322187
Genotyper Software v 3 7	Multiple licenses	4310700
Genotyper Software v 3 7	Upgrade	4316286
Genotyper Software v 3 7	For Macintosh®/Windows NT® system conversion	4310995

*Protocol not included

**First-time SNaPshot Multiplex users will need to purchase a multiplex kit, GeneScan-120 LIZ Size Standard, a matrix standard set appropriate for your instrument, and Genotyper software v 3.7. Additional purchase of matrix standard is required only if run conditions have changed. Please see your instrument's user manual for more information.

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ABI PRISM® SNaPshot™ Multiplex Kit Quick Reference Card

This quick reference guide is intended for use only by appropriately trained laboratory personnel who have read the user's manual and are experienced with the protocol. For safety guidelines, please refer to the *ABI PRISM® SNaPshot™ Multiplex Kit Protocol* (P/N 4323357). For all chemcials in bold type below, please read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Preparing the SNaPshot Control Reactions

Step	Action
1	Label two 0.2-mL MicroAmp® tubes: ♦ One for the positive control reaction ♦ One for the negative control reaction
2	Combine the following items in each tube.
3	Mix well, spin briefly, and immediately place on ice.

Item	Positive Control (µL)	Negative Control (µL)
SNaPshot Multiplex Ready Reaction Mix	5	5
SNaPshot Multiplex Control Primer Mix	1	1
SNaPshot Multiplex Control Template	2	0
Deionized water	2	4
Total	10	10

Preparing the SNaPshot Reactions

Step	Action
1	Combine the following ingredients on ice.
2	Aliquot 10 µL into each MicroAmp tube/well and immediately place on ice.

Item	One Sample (µL)
SNaPshot Multiplex Ready Reaction Mix	5
Pooled PCR products	3
Pooled SNaPshot primers	1
Deionized water	1
Total	10

Thermal Cycling the SNaPshot Reactions

Step	Action
1	Place the tubes in a GeneAmp® 9600 thermal cycler and set the volume to 10 µL.
2	Repeat the following for 25 cycles: 96 °C for 10 seconds 50 °C for 5 seconds 60 °C for 30 seconds **Note** The conditions can be modified to accommodate specific primers.
3	Rapid thermal ramp to 4 °C and hold until ready for post-extension treatment.
4	Continue with "Post-Extension Treatment," on the back of this card.

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Post-Extension Treatment

Step	Action
1	Add one of the following to the reaction mixture and incubate at 37 °C for 1 hour. 1.0 Unit of Shrimp AlkalinePhosphatase (SAP) or 1.0 Unit of Calf Intestinal Phosphatase (CIP)
2	Deactivate the enzyme by incubating at 75 °C for 15 minutes.
3	Place the sample at 4 °C.

Preparing the SNaPshot Products for the ABI PRISM 310 Genetic Analyzer, 3100 Genetic Analyzer, or 3700 DNA Analyzer

Step	Action
1	Add 9 µL of HI-DI formamide into each tube. **⚠ WARNING CHEMICAL HAZARD. Formamide** is harmful if absorbed through the skin and may cause irritation to the eyes, skin, and respiratory tract. It may cause damage to the central nervous system and the male and female reproductive systems, and is a possible birth defect hazard. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
2	Add 0.5 µL of SNaPshot products and 0.5 µL of GeneScan™120 LIZ™ size standard into each tube.
3	Vortex briefly and quick spin.
4	Denature the samples by placing them at 95 °C for 5 minutes.
5	Quick spin or tap the tubes or plates to bring liquid to the bottom of the tubes.
6	Place the samples at 4 °C until ready to load.
7	To run samples on the 310 Genetic Analyzer, verify that you have selected the following in the data collection software: a. GS STR POP-4 (1 mL) E5 module b. E5 matrix c. GeneScan-120 LIZ size standard (change the default color to orange) To run samples on the 3100 Genetic Analyzer, verify that you have selected the following in the data collection software: a. E5 dye set b. SNP36_POP4 module c. GeneScan-120 LIZ size standard Analysis (change the default color to orange) To run samples on the 3700 DNA Analyzer, verify that you have selected the following in the data collection software: a. E5 dye set b. Modified SNP1_1 POP5 module c. GeneScan-120 LIZ size standard Analysis (change the default color to orange)

Kit	Number of Reactions	Part Number
ABI PRISM® SNaPshot™ Multiplex Kit	100[a]	4323151
	1000[a]	4323154
	5000[a]	4323155

a. Contains Multiplex Control Template and Multiplex Control Primer Mix for 30 control reactions

Note The fluorescent dyes are assigned to the individual ddNTPs as follows:

ddNTP	Dye Label	Color of Analyzed Data
A	dR6G	Green
C	dTAMRA™	Black
G	dR110	Blue
T(U)	dROX™	Red

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